Evaluation of a Method for Preservation of Surface Water Samples for Analysis of Microbial DNA

BY

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THESIS

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This thesis is dedicated to my parents, Marijane and Garry Hammond, who have provided constant, unwavering support in all of my pursuits big and small. Their dedication and drive inspire me to always keep reaching for more.

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LIST OF ABBREVIATIONS

BSA	Bovine Serum Albumin
CDC	Centers for Disease Control and Prevention
DI	Deionized
ENT	Enterococci
EPA	Environmental Protection Agency
FIB	Fecal Indicator Bacteria
GFDL	Geophysical Fluid Dynamics Laboratory
IQR	Interquartile Range
MeB	Method Blank
mL	Milliliter
μL	Microliter
μm	Micrometer
MST	Microbial Source Tracking
NPS	Nonpoint Source
NTC	No-Template Control
PBS	Phosphate-Buffered Saline
qPCR	Quantitative Polymerase Chain Reaction
RT	Room Temperature
USGS	United States Geological Survey
WASH	Safe Water, Sanitation and Hygiene
WHO	World Health Organization

SUMMARY

As of 2021 nearly 30% of the world's population has inadequate access to clean water. Socioeconomic disparities often find low-income countries lacking in proper water management infrastructure, leaving billions without safe water for drinking, cooking, agriculture, hygiene, and other necessary tasks. Many of these resource-poor communities rely on surface water sources like rivers and lakes for daily use.

Transmission of waterborne illnesses is a major concern in water-stressed areas. Pathogenic species of viruses, bacteria, and parasites can be found in waters contaminated with human and animal waste. Fortunately, most waterborne diseases are preventable, but the number and variety of pathogens makes it difficult to monitor them directly. Water quality monitoring programs rely on fecal indicator bacteria, produced from the same sources as pathogenic species, as a sign of potential fecal contamination. The Environmental Protection Agency has relied on bacterial culture techniques to quantify and identify fecal indicator bacteria for decades, where samples are applied to a nutrient-enriched media to encourage bacterial growth. Important indicator species, like enterococci, produce a color change when grown on specific types of media, making it relatively easy to confirm their presence and report concentrations.

Despite its widespread use, bacterial culture does have its limitations. Any method that ultimately relies on human interpretation introduces a certain amount of uncertainty and subjectivity, and culture methods need a minimum of 18 hours to produce results. Given that results of these tests can often influence public health decisions alternative methods of detection may be preferable.

Molecular analysis techniques are both faster and more precise than bacterial culturing. Analyses like quantitative polymerase chain reaction can produce results in a matter of hours, and remove a measure of subjectivity by eliminating human interpretation. With PCR, very specific gene sequences can be identified and amplified from extremely small source concentrations, which can be quantified in real time. It can also help to track exact sources of a contamination, which can bolster public health efforts and aid in disease outbreak investigations.

Unfortunately, the collection and preservation of environmental water samples can hinder access to water quality testing. As it stands a minimum of 100mL of water needs to be collected for each sample, which then needs to be kept at or below specific temperatures to be considered valid representations of the source's microbial environment. If samples cannot be analyzed within six hours of collection they will need to be filtered and stored at -80^{o}C, including any time they may spend in transit to a lab capable of molecular analysis. These factors may restrict the number of samples that can be collected and what analyses are available, especially in resource-poor and rural areas.

Whatman FTA cards were originally designed for the analysis of tissue samples, but have since been studied for their potential application in the preservation of fecal and environmental samples. A very small sample volume is applied to the card, and following application bacterial cells lyse, and the released nucleic acid are meant to remain stable at room temperature without environmental degradation. Current literature has described the ability of Whatman cards to preserve lab-cultured mixed bacterial samples, clinical fecal samples, and parasite DNA from surface water samples, in some cases for up to 3 years at ambient temperature. This would indicate that Whatman FTA cards may be

useful alternatives to current cryopreservation methods for preserving environmental surface water samples for identification of water quality indicators, a scenario that has yet to be evaluated.

Because Whatman cards are compact, can carry four discrete samples, are easily stored and shipped, and can preserve samples for long periods of time without degradation, they may provide access to a better understanding of a community's microbial landscape. More samples may be able to be collected in previously inaccessible or underserved areas, bolstering public health efforts by directing attention to the most immediate concerns through a broader breadth of available molecular analyses.

This thesis evaluated Whatman cards for use in the preservation of microbial DNA from surface water samples for molecular analysis. Three trials were conducted: the first describing limits of detection, the second comparing the effect of storage method and condition on sample integrity, both with lab-prepared *E. faecalis* cells at known concentrations. The second trial was then replicated, with minor modifications, for the evaluation of wastewater effluent. Samples were prepared for filtration through polycarbonate filters, DNA extraction, and qPCR analysis performed according to current EPA methods, and Whatman cards were prepared and DNA extracted according to manufacturer recommendations. Statistical analysis was performed using SAS version 9.4.

After our data was determined to have a non-normal distribution, median and range were used to best describe these data. Broad views of data distribution showed consistency between the enterococci Ct values within each respective media type (polycarbonate filter samples stored at either ambient temperature of -80^{o}C, and Whatman card samples), with all sample concentrations and effluent samples taken into account. More specific views of each media type at each specific sample concentration

confirmed that each sample type was consistent with itself across time, with polycarbonate samples showing a slight upward trend in Ct as storage length approached 56 days. Using the Kruskal-Wallis nonparametric comparison test for group comparisons showed that Whatman cards had similar consistency within itself across sample concentrations and storage lengths to polycarbonate filter samples stored at -80^{o}C.

Based on this research, Whatman cards present an alternative method of microbial DNA preservation from surface water samples, with less limitations in sample handling and transport than current methods. Samples can be stored for at least two months at ambient temperatures without experiencing nucleic acid degradation, though compared to polycarbonate filters at room temperature, Whatman cards produce more consistent Ct values at concentrations of 1 x 10^4 cells/mL and above.

The results of this research are supported by previous studies showing that Whatman cards are a viable method for preserving DNA for molecular analyses, compared to both cryopreservation and other ambient temperature storage methods, and added new evidence that Whatman cards can be used for the storage of environmental surface water samples. Although further studies need to be done to confirm the place of Whatman cards in environmental sampling, this initial research makes us optimistic that Whatman FTA cards can be used to give researchers easier access to communities that were previously considered unreachable, or with limited water sampling and analysis resources. The ease of transport provided by Whatman cards creates more access to labs with molecular analysis capabilities, providing a more precise description of the unique microbial populations found in water-stressed communities. Understanding the source and type of microbial contamination can help guide public health efforts to build targeted water management and sanitation infrastructure to meet the needs of

the community, and help to ease the global burden of waterborne diseases by bolstering access to safe drinking to those that need it most.

1. INTRODUCTION

Access to clean drinking water is an ongoing global public health challenge. Many low-income countries lack water treatment infrastructure, leaving nearly 2.5 billion people worldwide with unreliable or no access to safe drinking water (World Health Organization [WHO], 2019a). People living in water-scarce areas often rely on wells or surface water for drinking, cooking, washing, agriculture, recreation, and other daily tasks. Improper waste management along with poor hygiene and sanitation practices can lead to the pollution of these drinking water sources, resulting in the spread of waterborne illnesses.

Safe water, sanitation and hygiene (WASH)-related diseases accounted for an estimated 1.9 million deaths in 2016 alone, and an average of 827,000 deaths annually (WHO, 2019b). Pathogenic viruses, bacteria, protozoa, and other parasites can be found in contaminated water, and can affect multiple body systems. Arguably the most well-known waterborne illnesses, cholera and dysentery, can lead to severe diarrhea; these and other diarrheal diseases are the second leading cause of death worldwide in children under 5 years old (Centers for Disease Control and Prevention [CDC], 2015). The burden of diarrheal diseases is disproportionately high in sub-Saharan Africa and south Asian countries where WASH infrastructure is lacking and other exacerbating health issues have a high prevalence (Black et al., 2003).

In addition to causing life-threatening acute illness, waterborne pathogens can also lead to chronic infection or long-term debilitation. Schistosomiasis, transmitted through dermal contact with contaminated water, can cause bladder cancer and internal bleeding as Schistosomes lay eggs within the body and cause irritation (Fenwick, 2012). When Schistosome eggs lodge in the intestinal wall they can compromise the ability of the individual to fight off other pathogens, and in rare cases the eggs of

certain Schistosome species can travel to the brain and cause chronic central nervous system symptoms (Verjee, 2019).

Waterborne diseases are not limited to developing countries. While most Americans may take clean water for granted thanks to government regulation, that safety is not guaranteed. Between 2000 and 2015 approximately 7.15 million people in the United States displayed clinical symptoms attributed to waterborne infections annually (Collier et al., 2021). High quality drinking water is a concern with aging water management infrastructure across the U.S.; nearly 43 million Americans rely on private well water with no federally mandated quality testing (U.S. Geological Survey [USGS], n.d.). High-income countries have also directed more attention to monitoring recreational water quality in recent decades, leading to greater recognition of chlorine-resistant pathogens like *Cryptosporidium* (Collier et al., 2021).

The global risk of waterborne illness is exacerbated by the ongoing climate crisis. Rising ocean and freshwater temperatures lead to areas that were previously considered low risk becoming far more hospitable habitats for pathogens. The Geophysical Fluid Dynamics Laboratory (GFDL) (2021) predicts that anthropogenic global warming will be directly responsible for an increase in both the number and severity of tropical cyclones in coming years, events that can lead to torrential rainfall and catastrophic flooding that can quickly overwhelm water management infrastructure. On the other end of the spectrum, extreme drought conditions can contribute to increased water scarcity, displacing populations into already taxed areas and decreasing overall water quality (Semenza, 2020). The current trajectory of the climate crisis combined with pre-existing vulnerabilities necessitates a robust water quality surveillance system to ensure that every person has access to the basic human right of clean water.

1.1 Literature Review

1.1.1 Current Water Monitoring Methods

Waterborne fecal pathogens consist of a wide range of organisms and can be released into the environment from a variety of sources. This makes detecting specific pathogenic species extremely difficult, relying predominantly on being at the right place at the right time to collect a representative sample. Instead of attempting to monitor pathogens directly, exposure risk is determined by the quantification of "indicator" species. Fecal indicator bacteria (FIB), such as *Escherichia coli*, coliforms, and enterococci, are naturally occurring in the intestines and fecal matter of humans and animals; as such, their presence in surface water can be considered a reasonable indication of fecal contamination and potential fecal pathogens (Motlagh & Yang, 2019).

Culture methods have been employed in the identification and quantification of bacteria since the late 1800's. These methods involve applying a small amount of material, such as surface water, onto a nutrient-rich media that encourages bacterial growth. After an incubation period typically lasting 24 to 72 hours bacterial colonies can be manually counted and species identified via Gram stain, specific media indicator, or other techniques (Environmental Protection Agency [EPA], 2002a). These methods are a relatively inexpensive way to determine the presence of and quantify target bacterial species from various sources, leading to their adoption as the standard of microbial water quality monitoring by the EPA. Enterococci, coliforms, and *E. coli* are readily grown in laboratory conditions, with EPA Methods 1600 (2002b) and 1604 (2002a) calling for the filtration of 100mL of surface water grab sample through a polycarbonate membrane filter to collect bacterial cells. That filter is then applied to either liquid or solid growth media, and after an incubation period of up to 24 hours can be inspected for the presence of bacterial colonies. Colonies of target species will undergo either a change in color (for *E. coli*), create a color change in the surrounding media (enterococci), or fluoresce under UV light (for coliforms) due to

the breakdown of media substrates by specific bacterial enzymes (EPA 2002a; EPA 2002b). This allows for the quick identification and relative quantification of these indicator species, which then allows the monitoring lab to advise local governing bodies of potential health risks.

FIB have been shown to predict the presence of enteric pathogens from both human and animal sources, giving monitoring entities a way to estimate the risk of illness from these pathogens (Motlagh & Yang, 2019; EPA, 2012). The density of enterococci in a given water source, defined as the number of bacterial cells per 100mL of water, can be directly translated to an estimated illness rate of 32 or 36 of 1,000 primary contact recreators (EPA, 2012). Put simply, past epidemiological studies have shown that once a threshold of 30 enterococci cells per 100mL is reached, a predictable number of people out of 1,000 who directly interact with that water source (swimming, surfing, etc.) will display symptoms of gastrointestinal illness (EPA, 2012). This is one of the reasons why many water quality monitoring programs rely on the detection and quantification of FIB, like enterococci, to inform water-related public health decisions. Culture methods are still the most widely employed method of detection for FIB, but they are somewhat hindered by their inherent subjectivity. There is no way to guarantee that every person will interpret bacterial growth, color change, or any other visual indicator the same way, leading to inconsistent reporting of results. More importantly, these methods require at least 18 hours of incubation. This time needed for bacterial growth can allow conditions of elevated health risk to persist.

Molecular analysis techniques offer a more modern alternative for bacterial quantification. Method 1609.1 (EPA, 2015) for detection of enterococci has surface water samples collected and filtered in the same fashion as samples prepared for culture. Once filtered, nucleic acid is extracted from the collected enterococci and purified for analysis via quantitative PCR (qPCR). Quantitative PCR recognizes specific nucleotide sequences in the genome of a target species and can create millions of

copies of target DNA, which can then be detected using fluorescent tagging to produce real-time growth curves (EPA, 2015). It takes approximately 4 hours for qPCR to produce results in comparison to at least 18 hours with culture methods.

Molecular analysis can also be used to identify what animal species is the source of fecal microbes in contaminated water through a method known as microbial source tracking (MST). Some portions of the microbial genome are conserved regardless of host, but some genes differ slightly depending on the animal host those bacteria are inhabiting. By recognizing this marker, host-associated qPCR can quantify FIB and identify their source simultaneously (EPA, 2018). This can help characterize the microbiological landscape in ways that allow public health efforts to focus on specific sources of fecal pollution. For instance, if MST determines that the enteric bacteria present at a specific Chicago beach are predominantly from dogs, nearby dog-friendly beaches can be investigated for waste management. This is also particularly useful in identifying nonpoint sources (NPS) of pollution. Waste from sewage systems is fairly easy to recognize, but NPS pollution typically comes from rain, melting snow or floods carrying pollutants from land into surface water (EPA, 2018). This makes identifying the source of those contaminants especially difficult when there's an outbreak of GI illnesses. A common example of NPS pollution is runoff from agricultural areas carrying fecal bacteria from livestock into surface water (EPA, 2018). MST can identify those bacteria as coming from cattle, pigs, chickens, etc., and investigations can then be conducted into nearby farmland. Once identified that source of pollution can be mitigated or eliminated, thereby lowering the overall risk of illness from affected waters.

Quantification and identification of FIB are also much more precise when using molecular methods, as PCR recognizes unique markers for each target species and creates real-time exponential growth curves, removing a large amount of subjectivity from the process. Even though qPCR alone

cannot determine the presence of live bacteria, as it only recognizes the presence or absence of bacterial nucleic acid, the speed of the analysis combined with its precision makes molecular methods more favorable than culture.

1.1.2 **Environmental Microbiology Challenges in Resource-Poor Areas**

While molecular analysis is one of the primary approaches in identifying fecal contamination in water, the instruments and reagents involved are cost prohibitive to many water monitoring programs, particularly in low-income countries. A thermocycler designed for quantitative PCR analysis can cost $20,000 USD or more. Reaction reagents (master mix, primers and probes, etc.) can cost $0.82 USD per reaction, or $78.72 USD for a typical full 96-well reaction plate, on average (Biosearch Technologies, 2015). Consumables needed for DNA extraction and qPCR analysis can add additional costs of approximately $75.00 USD to prepare samples to fill one 96-well plate, after averaging costs of nuclease-free tubes, assay controls, etc. from three different manufacturers. Altogether, after capital expenses, it can cost upwards of $40,000 USD per year to run one 96-well reaction plate five days a week, every week, not including the additional cost of hiring qualified personnel.

Another barrier to conducting molecular analyses of water samples collected in low-income countries are sample handling requirements. Water samples of 100mL are to be kept at 10^{o}C or less and analyzed within 6 hours from collection to ensure that results are representative of environmental bacterial populations, or otherwise filtered and frozen at -40^{o}C until ready for analysis (EPA, 2013). Access to adequate cooling and timely, reliable transportation to a lab where samples can be processed can become difficult to find when collecting environmental samples for water quality analysis. This is especially true in resource-poor and rural areas.

In low-income countries, where lack of access to clean water is especially prevalent, field samples may need to travel many hours to the nearest urban center - or even overseas - to find a lab capable of molecular analysis. Fluctuating temperature during travel or unsecured sample containers can compromise the integrity of the DNA in the sample, making sample preservation a challenge, and the costs of shipping on ice can be barriers to seeking such analyses in the first place. This may restrict the number of samples that can be collected, or the communities that can be reached.

1.1.3 **Whatman® FTA® Card Usage**

Whatman FTA cards (MilliporeSigma, Burlington, MA) are designed to isolate and stabilize nucleic acid from a variety of cell and sample types (Millipore Sigma, n.d.b.). Several different variations of Whatman FTA cards exist, including FTA Micro and Mini Cards with room for one or two samples respectively, color-changing indicator cards useful for use with clear samples, and cards designed for extremely small sample volumes. Whatman FTA Classic cards (referred to as Whatman FTA cards or Whatman cards in this thesis) are by far the most widely used and well-studied for their application in molecular analyses, including use for preserving environmental samples. These cards have spaces in which four individual 100μL samples can be applied. Following application, cells lyse, and the released nucleic acid are meant to remain stable at room temperature without environmental degradation (Millipore Sigma, n.d.).

It has been demonstrated that mixed bacterial samples can be preserved on Whatman cards for at least three years at ambient temperature, after which real-time PCR analysis was able to successfully detect several important rRNA targets at bacterial concentrations as low as 10^{-1} cells/mL (Rajendram et al., 2006). Comparisons of the effects of storage method on mixed bacterial cultures have also been conducted, showing recovery rate and quality of DNA extracted from Whatman cards is comparable to

other common storage methods, though it may have an effect on which parts of the bacterial community are best represented (Song et al., 2016). In particular, Whatman cards seem to be less effective at preserving gram-negative bacteria (Rajendram et al., 2006). A 2018 review of studies comparing Whatman cards to other nucleic acid preservation methods by da Cunha Santos concluded that nucleic acid from a variety of sources can be reliably stored and extracted from the cards for use in molecular assays, providing further support for the use of Whatman FTA cards for microbial DNA preservation as an alternative to cryopreservation.

Originally designed for analyzing human tissue and blood samples, Whatman cards have since been employed in the storage of microbial DNA from both human fecal and surface water samples. In a comparison with other ambient temperature nucleic acid preservation methods for PCR analysis, fecal samples were applied directly to each medium, with Whatman cards performing similarly to all other methods as defined by DNA recovery efficiency (Nechvatal et al., 2008). Lalani et al. (2018) conducted a study of human fecal matter applied and stored at ambient temperature on Whatman cards to the same fecal matter stored frozen and similarly found that recovery of bacterial DNA and identification of specific targets from samples stored on Whatman cards performed similarly to, if not better than, the frozen samples. Whatman cards have also been examined for their performance in storing microbial DNA from surface water samples, finding them to be a potential alternative for the preservation and identification of lab-cultured replicates of marine bacterial communities (Gray et al., 2012). Another study used Whatman cards to collect and store surface water samples from rivers in China to detect DNA from *Schistosoma japonicum*, a waterborne trematode that can cause Schistosomiasis. This study concurred with other studies that high quality DNA could be reliably extracted from Whatman FTA cards for PCR analysis (Worrell et al., 2011). These studies lead to the reasonable assumption that Whatman

FTA cards may be useful alternatives for preserving environmental surface water samples for identification of water quality indicators, a scenario that has yet to be fully evaluated.

Use of Whatman cards in testing environmental surface water would remove many of the limitations associated with sample collection and preservation. The cards are compact, measuring 3 x 5 inches, and can fit 4 unique 100μL samples each. Once samples are collected, they can be stored at ambient temperature with no specific handling requirements, meaning that a large number of samples can be collected, transported and analyzed more or less at the ease of the researcher. This would allow for more thorough monitoring of water quality in difficult to reach areas by allowing for the collection of a higher volume of samples and access to a larger number of labs for support in processing and analysis due to ease of transport. Better understanding of a community's microbial landscape can bolster public health efforts by directing attention to the most immediate concerns, whether that be sources of fecal pollution, identification of high-risk populations, or implementation of the correct water treatment methods. A broader breadth of studies can more easily be conducted, such as assisting in rapid disease outbreak investigations and more precise MST by providing easier access to molecular labs. Establishing the use of Whatman cards in environmental surface water sampling may also open opportunities for application to environmental soil, dust, or marine samples, further enabling more robust public health efforts.

1.2 **Study Objective**

This research is attempting to evaluate Whatman FTA cards as a potential alternative to current bacterial DNA preservation methods for molecular analysis. Standardized concentrations of *E. faecalis* and wastewater effluent were applied to Whatman cards and filtered through polycarbonate filters according to EPA Method 1609 (2013) and 1609.1 (2015). Samples were then stored for varying lengths

of time and storage conditions before preparation for qPCR analysis. Performance of each media was ultimately determined by the efficiency of DNA extraction, quality of extracted DNA, and accuracy of qPCR quantification.

2. METHODS

2.1 Study Description

This thesis evaluated Whatman FTA cards (MilliporeSigma, Burlington, MA) as a possible method of collecting and storing surface water samples for analysis of microbial DNA. Three trials were conducted to better describe how Whatman cards perform in comparison to the current polycarbonate filter method described in EPA Method 1609 (EPA, 2013) and 1609.1 (EPA, 2015). Performance, for the purpose of this thesis, is defined as the quality and recovery rate of microbial DNA from each media type as quantified by qPCR analysis. The objectives of each trial are as follows:

1. A comparison of the DNA integrity and recovery of a known concentration of *Enterococcus faecalis* cells from polycarbonate filters and Whatman cards.
2. A comparison of the performance of polycarbonate filters and Whatman cards for preserving microbial DNA from known concentrations of *E. faecalis* cells at different storage lengths and under varying storage conditions.
3. A comparison of the performance of polycarbonate filters and Whatman cards for preserving microbial DNA from wastewater effluent at different storage lengths and under varying storage conditions.

2.2 Sample Preparation

The following analyses were prepared following EPA's Method 1609 (EPA, 2013) and 1609.1 (EPA, 2015) unless otherwise noted. In brief, samples (volumes specified below) were filtered through 47mm hydrophilic polycarbonate filters with a pore size of 0.4μm (MilliporeSigma, Burlington, MA) before transfer to individual nuclease-free 1.5mL pre-filled glass bead tubes (GeneRite, North Brunswick, NJ). Bacterial DNA was then extracted from filters by bead-beating and suspended in a Sketa-22 salmon DNA external control (described in EPA Method 1609 [EPA, 2013]), used to help ensure the quality of the

DNA extraction. A 5μL volume of final DNA product was then added to 20μL of prepared master mix solution, which consisted of primers and probes as described in EPA Method 1609 (EPA, 2013) (Integrated DNA Technologies, Coralville, IA), TaqMan® Environmental Master Mix (Applied Biosystems, Waltham, MA), bovine serum albumin (BSA), and nuclease-free PCR-grade water; in an alteration of EPA methods, no internal amplification control was used. All qPCR analyses were performed using the Applied Biosystems QuantStudio™ 3 real-time PCR system.

All Whatman card samples were prepared by holding the interior nucleic acid capture card up to separate it from the outer card backing and prevent sample leak through. Prepared *E. faecalis* cell dilutions and effluent samples were thoroughly vortexed for 30 seconds, or shaken by hand 30 times for larger volumes, before 100μL of sample were pipetted directly into the center of each Whatman card sample area. The top card cover was then used to prop the inner nucleic acid capture card up as the cards were allowed to air dry for 25-30 minutes. After each card was completely dry samples were removed from the card using a sterilized razor blade and transferred to individual 1.5mL low-retention nuclease-free tubes (Sarstedt Inc., Nümbrecth, Germany), or otherwise stored in a plastic bag with a silica desiccator at ambient room temperature. Bacterial DNA was extracted from Whatman card samples using the QIAmp DNA Investigator Kit (Qiagen, Hilden, Germany) with the following protocol change: QIAprep spin columns were used in place of minElute spin columns provided with the kit due to improper storage of the minElute columns upon receipt. This protocol utilizes a supplied lysis buffer, spin columns with silica membranes to capture and purify bacterial DNA from the lysate, and sterile PCR-grade water to elute the final DNA product. These samples were analyzed via qPCR using the same method as described above for polycarbonate filter samples.

2.2.1 **Recovery and Range Finding Trial**

To describe the difference in recovery rates of microbial DNA between polycarbonate filters and Whatman FTA cards, *E. faecalis* calibrator cells (cultured in-lab and prepared according to EPA method 1609.1 [EPA, 2015]) were serial diluted from a starting concentration of 10^6 cells/mL to create standards of known concentrations ranging from 1 x 10^1 cells/mL to 1 x 10^5 cells/mL (Recovery and Range Finding Trial Protocol, Appendix A). Each sample was filtered as described in section 2.2, and consisted of 99mL sterile deionized (DI) water and 1mL of the appropriate *E. faecalis* standard to reach the desired final concentration, for a total volume of 100mL. A method blank (MeB) of 30mL sterile phosphate-buffered saline (PBS) was filtered at the same time. Whatman cards were prepared according to the method described in section 2.2, with 100μL of the appropriate standard applied to the card to reach the desired final concentration, and with an MeB of 100μL of PBS.

2.2.2 **Storage Trial**

Multiple "sample sets", all consisting of identical samples (except for day 0 which did not include frozen polycarbonate samples), were prepared concurrently to be analyzed at set time intervals. Standard stock solutions of *E. faecalis* cells were prepared in the same manner as described in section 2.2.1. The Storage Trial Protocol (Appendix A) describes how many samples were prepared at each concentration by media type, storage condition, and storage length. Polycarbonate filter samples and Whatman card samples were prepared as described in section 2.2, with respective MeB controls as described in section 2.2.1. Samples on polycarbonate filters were stored at either room temperature or -80^{o}C per EPA Method 1609.1 (EPA, 2015). All Whatman card samples were stored at ambient room temperature per manufacturer instruction.

2.2.3 **Effluent Trial**

Effluent samples were prepared for filtration and Whatman card analysis concurrently with Enterolert® samples. Samples of 100mL of effluent were filtered through a 45mm polycarbonate filter with a 0.4μm pore size, along with a method blank (MeB) of 20mL sterile PBS and a positive control of 1mL 10^6 cells/mL *E. faecalis* cells. Each filter was then placed into an individual nuclease-free 1.5mL glass bead tube. Whatman cards were prepared according to the method described in section 2.2. Number of samples prepared for each media type, storage conditions, and storage lengths can be found in Effluent Trial Protocol (Appendix A).

2.3 **DNA Extraction and qPCR Analysis**

Bacterial DNA extraction of polycarbonate filter samples and qPCR analysis were done according to EPA Methods 1609 (EPA, 2013) and 1609.1 (EPA, 2015) with one exception. No plasmid internal amplification control was used, though salmon DNA sample processing control was. Use of the QIAmp DNA Investigator Kit for Whatman DNA extraction was recommended by the manufacturer in the Reliable DNA extraction from Whatman FTA cards study (MilliporeSigma, n.d.a.), which also supplied the protocol.

2.4 **Data Quality Assurance**

Data quality was ensured following the protocol described in EPA Methods 1609 (EPA, 2013) and 1609.1 (EPA, 2015). Method blanks consisted of 30mL of sterile PBS for polycarbonate filtration, and 100μL of sterile PBS for Whatman cards. Calibrator cells consisting of 1mL of lab-cultured, 1 x 10^6 cells/mL *E. faecalis* cells were filtered through polycarbonate filters as a positive control in the effluent trial. Both method blanks and calibrator cells underwent DNA extraction using the same procedure as

the other samples, and were loaded onto the same PCR plates along with a no-template control (NTC). All controls and all samples were run in duplicate.

Samples were prepared in a working "dirty" lab, where surface water samples are received and processed. Bacterial DNA extraction occurred in a separate lab dedicated for that purpose. Benchtops in both labs were cleaned with bleach, which was allowed to sit on the bench surface for 5 minutes, and 70% isopropyl ethanol, before and after use. Master mix for qPCR analysis was made in a dedicated "clean" hood, which was thoroughly cleaned with 70% isopropyl ethanol and RNase AWAY® Reagent (Thermo Fisher Scientific, Waltham, MA) before and after use.

Standard curves for the QuantStudio 3 system were generated using EPA-supplied standards, with each standard run in triplicate. Results were compared to previously established Ct thresholds for accuracy.

2.5 **Statistical Analysis**

Statistical analyses were conducted using SAS version 9.4 (SAS Institute Inc., Cary, North Carolina). Enterococci Ct values, even within a given storage media type and for a given enterococci starting concentration, were not distributed normally based on the Kolmogorov-Smirnov test ($p < 0.0010$). Attempts to log10 transform individual data sets to establish a normal distribution were unsuccessful. Sample median and range were used in place of mean and standard deviation to better describe these data. Comparisons between groups were conducted using the Kruskal-Wallis nonparametric comparison test, using $p \geq 0.05$ to indicate no statistically significant difference. Box plots were generated in SAS from the same data set. Any transformation of observations was also done in SAS.

2.6 **Conflict of Interest Statement**

The author would like to state that neither she nor anyone involved in this thesis have any financial, proprietary, or other relevant interests relating to the research conducted.

3. RESULTS

A total of 404 samples from all trials and media types were analyzed, with 252 samples filtered through 47mm polycarbonate filters and 152 individual Whatman FTA card samples. Of the polycarbonate filter samples, 69 were extracted immediately after filtration, 93 were stored at -80°C, and 90 were stored at ambient room temperature. A summary of the number of samples analyzed per trial, by storage length, and by concentration can be found in Table I.

TABLE I

SAMPLE FREQUENCY BY MEDIA TYPE, STORAGE TEMPERATURE, AND STORAGE CONDITION

Media Type	**n**			
Polycarbonate Filter	252			
Whatman Card[a]	152			
		Frequency (n), Media Type and Storage Condition		
Cell Concentration (cells/mL)		**Poly., RT[b]**	**Poly., -80°C[c]**	**Whatman Card**
1×10^1	72	27	18	27
1×10^2	78	30	18	30
1×10^3	84	33	18	33
1×10^4	84	33	18	33
1×10^5	6	3	0	3
Effluent	80	33	21	26
TOTAL	**404**	159	93	152
Storage Length (Days)				
Immediate Extraction	132	69	0	63
24 hours	101	33	36	32
7 days	81	27	27	27
14 days	9	3	3	3
28 days	45	15	15	15
56 days	36	12	12	12
TOTAL	**404**	159	93	152

[*] N = 404.
[a] A Whatman card sample is defined as a single 1-inch sampling area, with 4 sampling areas per card.
[b] Polycarbonate filter samples stored at ambient room temperature.
[c] Polycarbonate filter samples stored at -80°C.

Some items of note from Table I include samples at day 14, which only consist of effluent samples. This trial added a sample point at 14 days due to time constraints necessitating elimination of a sample point of day 56, meaning that only samples with known concentrations are represented at that time. A sample concentration of 1 x 10^5 was only evaluated in trial 1 and subsequently dropped from later trials, accounting for the limited sample size.

Enterococci (ENT) Ct values grouped by concentration, based on the Kolmogorov-Smirnov statistic, did not follow a normal distribution ($p < 0.0010$, $\alpha = 0.05$). This was also true for subsets of the data defined by enterococci cell concentration. Visual inspection of associated histograms and Q-Q plots presented the effluent data points in a subjectively near-normal distribution, but could not be normalized after manipulation with a log transformation (Table II). ENT Ct medians followed an expected trend of decreasing Ct with increasing cell concentration, with a Ct difference of approximately 2 to 3 between median Ct values for each concentration, consistent with a 10-fold dilution. Effluent samples and samples with a concentration of 1 x 10^4 cells/mL had the largest Ct ranges of 17.57 and 16.60 respectively, though interquartile range (IQR) was both similar and relatively low (approximately 2.50) across all samples apart from effluent (IQR 11.57) (Table II). A Ct value of 40, representing nondetectable concentrations (nondetects) for this assay, also represent the 95th percentile for effluent samples and samples with a concentration of 1 x 10^2 cells/mL, which is especially noteworthy given the large range and IQR for effluent.

TABLE II

DESCRIPTIVE STATISTICS AND TESTS FOR NORMAL DISTRIBUTION BY CONCENTRATION FOR ENTEROCOCCI CT

Concentration (cells/mL)	**n**	**Kolmogorov-Smirnov (p-value)**	**Shapiro-Wilk (p-value)**	**Median (5th, 95th)**	**Range**	**IQR**[**]	**Skewness**	**Kurtosis**
1×10^2	78	<0.0100	<0.0001	34.87 (33.51, 40.00)	7.31	2.26	1.13	0.43
1×10^3	84	<0.0100	<0.0001	32.45 (30.97, 35.89)	8.82	2.57	1.04	1.03
1×10^4	84	<0.0100	<0.0001	29.03 (23.74, 32.56)	16.60	2.58	0.17	2.01
Effluent	80	<0.0100	<0.0001	27.02 (23.68, 40.00)	17.57	11.75	0.41	-1.49
Effluent (log10)[a]	80	<0.0100	<0.0001	N/A	N/A	N/A	0.30	-1.59

[*] At α = 0.05.

[**] IQR = interquartile range.

[a] Effluent (log10) refers to effluent sample mean Enterococci Ct values that have been log10 transformed.

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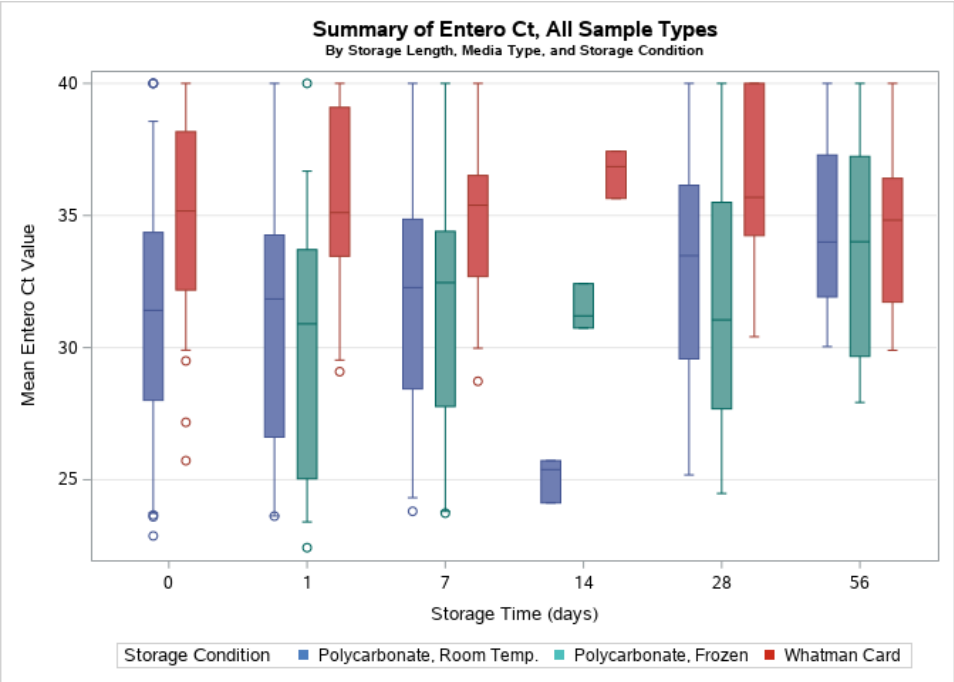

Figure 1. Visualization of the distributions of enterococci Ct values for all sample types and concentrations, grouped by storage length. Whiskers represent 5th and 95th percentiles, and circles represent outliers. Hash mark within each box represents the median.

A broad overview of combined ENT Ct from all sample concentrations (including effluent samples) shows relative consistency within each storage condition across all storage lengths (Table III, Figure 1). These data also highlight the large ranges seen in Table II, visualizing the effect of Whatman effluent Ct values on summarized Whatman Ct distributions. However, Whatman sample Ct values are very close at each time point regardless of whether effluent samples are included in the data (14 days) or absent (56 days, which did not include effluent samples), despite effluent samples seeming to account for most outliers. Similarly, the data does not show a significant difference in performance between polycarbonate filters stored at ambient room temperature and polycarbonate filters stored at -80^{o}C, with the only notable exception being the effluent samples evaluated at 14 days. The particularly

large gap in Ct distributions between frozen polycarbonate effluent samples and those stored at room temperature at this time point is inconsistent with the distributions seen at days 7 and 28.

TABLE III

MEDIAN ENTEROCOCCI CT BY MEDIA, STORAGE CONDITION, AND STORAGE LENGTH

	Median Enterococci Ct (5th, 95th)					
Storage Time	**n**	**Polycarbonate, RT**	**n**	**Polycarbonate, -80°C**	**n**	**Whatman Card**
Immediate	39	31.20 (23.60, 40.00)		N/A	33	35.62 (30.16, 40.00)
24 hours	33	31.84 (23.63, 40.00)	36	30.90 (23.40, 36.68)	32	35.11 (29.53, 40.00)
7 days	27	32.27 (24.32, 40.00)	27	32.46 (23.80, 40.00)	27	35.39 (29.98, 40.00)
14 days[a]	3	25.39 (24.12, 25.72)	3	31.20 (30.74, 32.42)	3	36.85 (35.64, 37.43)
28 days	15	33.48 (25.18, 40.00)	15	31.05 (24.48, 40.00)	15	35.69 (30.41, 40.00)
56 days[b]	12	34.00 (30.04, 40.00)	12	34.01 (27.93, 40.00)	12	34.82 (29.90, 40.00)

[a] Only effluent samples were analyzed at day 14.
[b] No effluent samples were analyzed at day 56

Further grouping the data by sample type (known concentration or effluent) as well as storage condition and length provides a more specific view, and the expected downward trend in median Ct as cell concentration increases can once again be observed (Table IV). This is apparent at all storage lengths and all storage conditions, with an occasional loss of distinction between ENT Ct values of samples at concentrations of 1×10^1 and 1×10^2 cells/mL (Table IV). Nondetects (represented by Ct values of 40) are more prevalent at lower concentrations, representing 17.20% of all polycarbonate samples and 26.32% of all Whatman card samples at concentrations of 1×10^2 and below. A total of 34.62% of Whatman effluent samples are also at a Ct of 40 compared to 3.33% of polycarbonate effluent samples.

TABLE IV

MEDIAN ENTEROCOCCI CT VALUES BY CELL CONCENTRATION AND STORAGE CONDITIONS OVER TIME

Storage Time	Concentration (cells/mL)	Median (5th, 95th) Enterococci Ct		
		Polycarbonate Filter	Whatman Card	
Immediate				
	1×10^1	34.44 (33.47, 40.00)	39.09 (36.28, 40.00)	
	1×10^2	34.56 (33.51, 36.11)	35.02 (34.04, 36.81)	
	1×10^3	31.33 (30.93, 32.20)	34.03 (32.18, 35.30)	
	1×10^4	25.85 (25.60, 28.05)	30.51 (29.50, 31.52)	
	Effluent	25.85 (22.88, 40.00)	37.31 (34.85, 40.00)	
		Polycarbonate Filter Samples by Storage Temperature		
24 hours		**Frozen (-80°C)**	**Room Temperature**	**Whatman Card Samples**
	1×10^1	35.45 (33.20, 40.00)	35.71 (33.20, 40.00)	39.23 (36.80, 40.00)
	1×10^2	33.85 (33.40, 34.63)	34.41 (33.81, 35.21)	34.50 (33.68, 40.00)
	1×10^3	31.45 (30.25, 32.67)	31.51 (30.89, 32.24)	33.81 (32.35, 34.04)
	1×10^4	25.95 (23.40, 28.23)	27.89 (23.62, 40.00)	29.92 (29.09, 31.64)
	Effluent	25.04 (22.43, 32.20)	25.85 (23.63, 37.04)	36.69 (35.36, 40.00)
7 days				
	1×10^1	35.19 (33.74, 40.00)	35.30 (33.70, 40.00)	36.71 (35.39, 38.34)
	1×10^2	34.38 (34.00, 34.76)	35.47 (33.81, 35.47)	35.99 (32.99, 40.00)
	1×10^3	31.66 (31.14, 32.53)	32.17 (31.60, 32.31)	34.92 (33.27, 35.89)
	1×10^4	25.85 (23.74, 27.84)	26.57 (23.81, 29.73)	30.47 (28.73, 32.56)
	Effluent	24.40 (24.35, 25.06)	24.86 (24.32, 24.88)	40.00 (35.36, 40.00)
14 days				
	Effluent	31.20 (30.74, 32.42)	25.39 (24.12, 25.72)	36.85 (35.64, 37.43)
28 days				
	1×10^1	40.00 (35.78, 40.00)	36.98 (35.10, 40.00)	40.00 (35.69, 40.00)
	1×10^2	34.72 (34.52, 35.50)	36.14 (35.19, 40.00)	36.49 (35.59, 40.00)
	1×10^3	31.05 (30.97, 31.12)	33.48 (32.85, 33.84)	35.50 (34.72, 35.95)
	1×10^4	27.00 (27.68, 27.71)	29.88 (29.57, 30.02)	31.20 (30.41, 34.24)
	Effluent	24.89 (24.48, 25.33)	25.57 (25.18, 27.10)	40.00 (33.98, 40.00)
56 days				
	1×10^1	40.00 (37.12, 40.00)	40.00 (39.31, 40.00)	36.31 (35.98, 40.00)
	1×10^2	36.67 (36.41, 37.34)	34.13 (33.86, 35.26)	36.51 (36.00, 37.24)
	1×10^3	31.18 (31.17, 31.61)	33.13 (32.45, 34.30)	32.86 (32.58, 33.66)
	1×10^4	27.95 (27.93, 28.17)	30.89 (30.04, 31.39)	30.50 (29.90, 30.86)

Even with potential outliers, both Whatman card and polycarbonate samples appear to have consistent Ct values for each sample concentration across all storage lengths. An exception is polycarbonate filters at both RT and -80°C showing a slight increase in Ct the longer samples are stored, a trend visible starting at day 28 (Figure 2, Figure 3). This is particularly evident at a concentration of 1 x 10^4 cells/mL (Table IV, Figure 3). Whatman samples, while continuing to display a slightly higher Ct value at all points when compared to polycarbonate, are also apparently more consistent at each storage length, especially at higher concentrations (Table IV).

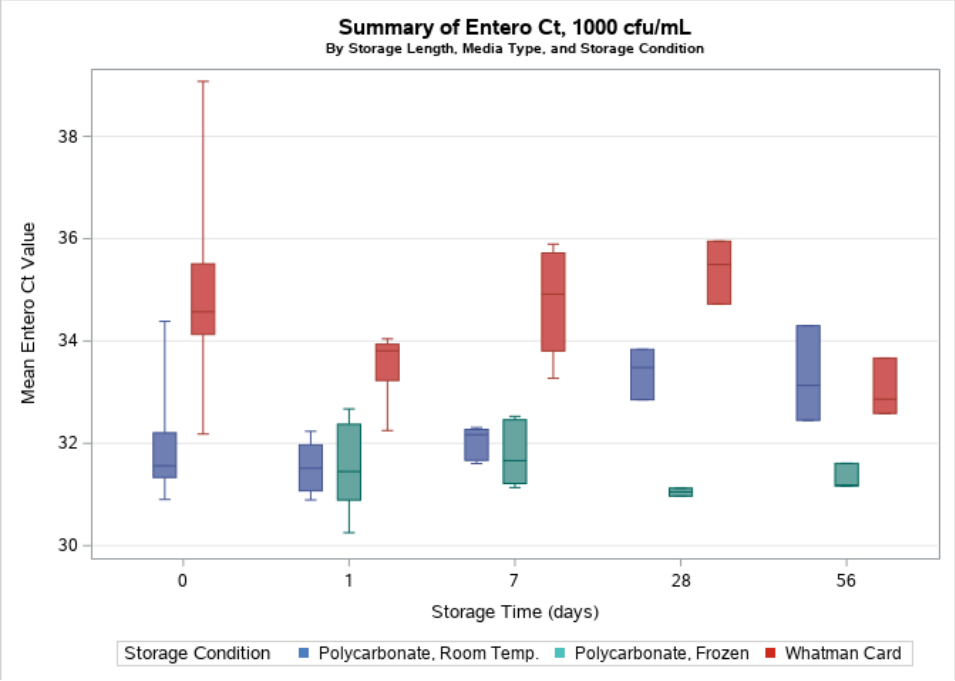

Figure 2. Visualization of distribution of enterococci Ct values for samples at a concentration of 1 x 10^3 cells/mL, grouped by storage length. Whiskers represent 5^{th} and 95^{th} percentiles. Hash mark within each box represents the median.

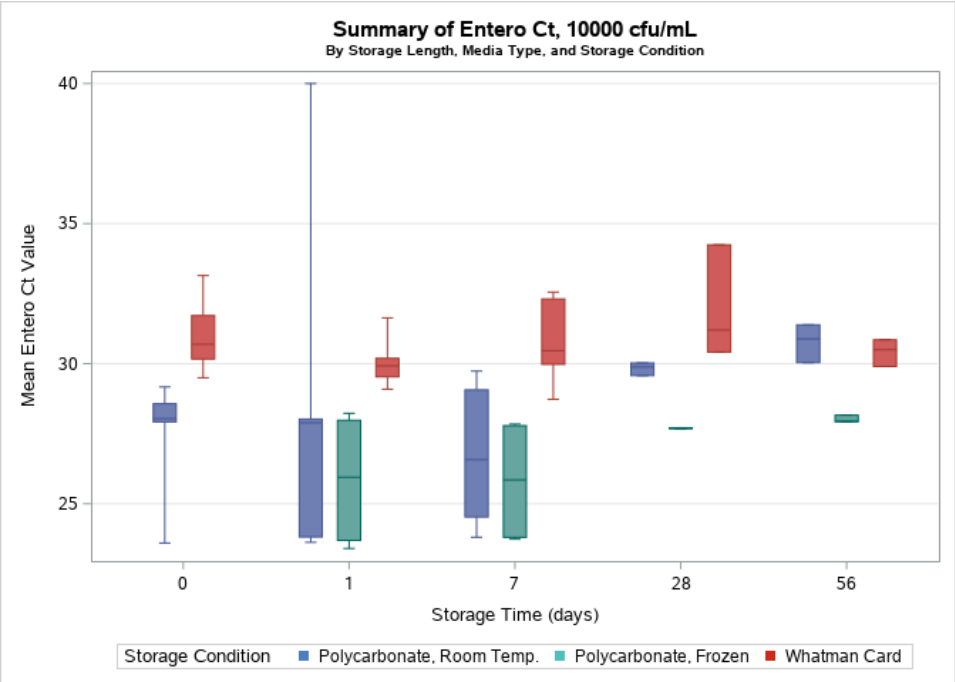

Figure 3. Visualization of the distribution of enterococci Ct values for samples at a concentration of 1 x 10^4 cells/mL, grouped by storage length. Whiskers represent 5^{th} and 95^{th} percentiles. Hash mark within each box represents the median.

Effluent samples returned the least uniform results for both media types, consistent with the large enterococci Ct range seen in Table II. As touched on previously, much of this range can be explained by the large gap between polycarbonate and Whatman Ct values (Figure 4), along with an apparent outlier for polycarbonate samples stored at -80°C at day 14 (Table IV). As Whatman card samples are 100µL each compared to 100mL filtered through polycarbonate this large Ct gap is not unexpected, and is further amplified by small sample size.

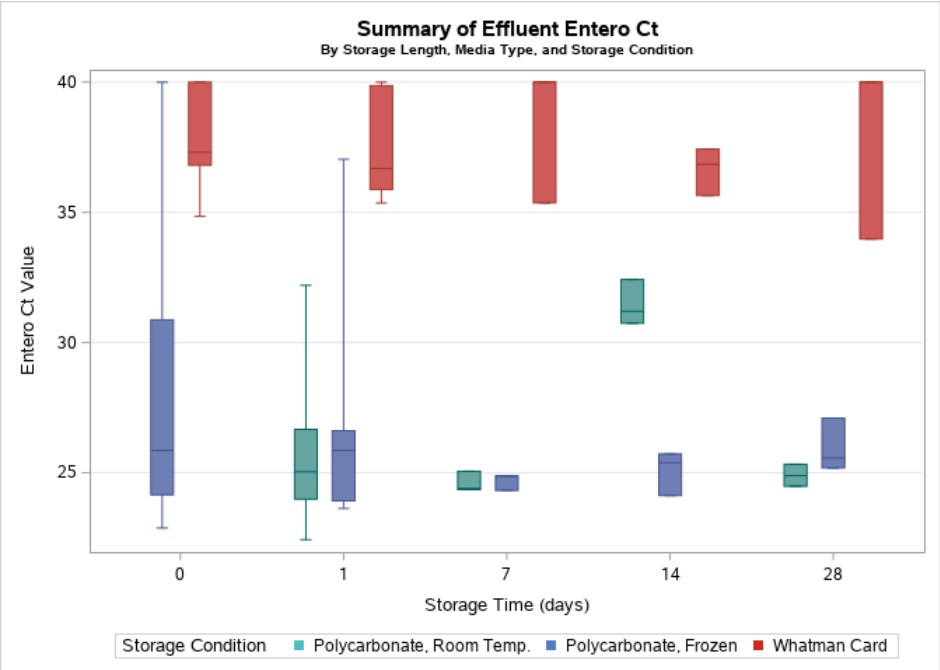

Figure 4. Visualization of the distribution of enterococci Ct values for effluent samples, grouped by storage length. Whiskers represent 5th and 95th percentiles. Hash mark within each box represents the median.

To attempt to compensate for the large difference in sample volume between polycarbonate and Whatman effluent samples, Whatman Ct values for this data set were adjusted by subtracting 9.97, the approximate Ct equivalent to a 1000-fold increase or decrease in sample concentration. Whatman effluent samples with a Ct of 40 were not adjusted and excluded from further analyses for both media types. New descriptive statistics were generated and showed that these data still do not follow a normal distribution (Kolmogorov-Smirnov $p < 0.0010$, $\alpha = 0.05$) (Table V). The new median ENT Ct for Whatman effluent samples is 27.18, down approximately 3 Ct from the previous median and much closer to the median polycarbonate ENT Ct for each storage condition. A visual analysis of the adjusted data (Figure 5) confirms that, when compensating for the dilution factor, Whatman card effluent samples were consistent with polycarbonate effluent samples.

TABLE V

NEW DESCRIPTIVE STATISTICS AND KRUSKAL-WALLIS COMPARISON FOR ADJUSTED WHATMAN EFFLUENT CT VALUES

Storage Condition and Media	n	Kolmogorov-Smirnov Statistic (p-value)	Shapiro-Wilk Statistic (p-value)	Median (5th, 95th)	Range	IQR	Kruskal-Wallis Comparison (p-value)
Polycarbonate, RT	33	<0.0100	<0.0001	25.56 (23.64, 37.04)	17.13	2.95	
Polycarbonate, -80°C	21	<0.0100	0.001	25.06 (23.68, 32.20)	9.99	2.60	0.0174
Whatman Card[a]	26	<0.0100	0.002	27.18 (24.88, 30.03)	6.03	3.68	

[*] At α = 0.05.

[a] Whatman card sample Ct values have been adjusted down by 9.97 to compensate for 1000-fold dilution factor when compared to polycarbonate samples. All other Ct values are unchanged.

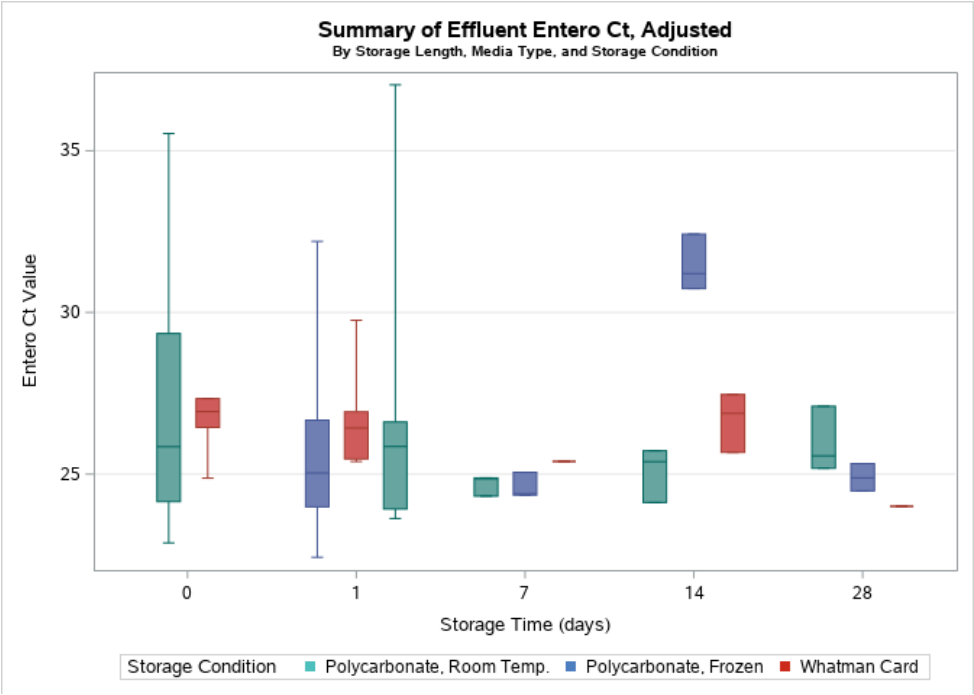

Figure 5. Visualization of enterococci Ct values for effluent samples, with Ct adjusted down by 9.97 for Whatman samples to compensate for dilution vs. polycarbonate and grouped by storage length. Whiskers represent 5th and 95th percentiles, and circles represent outliers. Hash mark within each box represents the median.

Comparison between group medians and trend analyses were conducted via the Kruskal-Wallis comparison test for nonparametrically distributed data (Table VI), with more in-depth initial results summarized in Tables VIII and IX, Appendix B. Based on these results we can see a significant difference in ENT Ct between each media and storage condition for effluent samples (all $p < 0.05$, $\alpha = 0.05$) (Figure 2). That difference is also present for samples at concentrations of 1 x 10^3 and 1 x 10^4 cells/mL (Figures 3 and 4), though at 24 hours there is no apparent significant difference at a concentration of 1 x 10^4 cells/mL ($p = 0.1821$, $\alpha = 0.05$). The ENT Ct for all samples at concentrations of 1 x 10^2 appear to have the least significant difference between them, which subjectively appears to be true when examining median ENT Ct values (Table IV; Figure 6, Appendix B).

TABLE VI

INITIAL KRUSKAL-WALLIS COMPARISON TEST RESULTS FOR SINGLE CONCENTRATIONS AND TIME POINTS[a]

Storage Length	Medium	1 x 10^2 cells/mL		1 x 10^3 cells/mL		1 x 10^4 cells/mL		Effluent	
Immediate		**n**	**p-value**	**n**	**p-value**	**n**	**p-value**	**n**	**p-value**
	Poly., RT	12	0.0522	15	<0.0001	15	<0.0001	15	<0.0001
	Whatman	12		15		15		9	
24 hours									
	Poly., RT	6		6		6		9	
	Poly., -80°C	6	0.1267	6	0.0003	6	0.1821	12	<0.0001
	Whatman	6		6		6		8	
7 days									
	Poly., RT	6		6		6		3	
	Poly., -80°C	6	0.3048	6	<0.0001	6	0.0027	3	<0.0001
	Whatman	6		6		6		3	
14 days									
	Poly., RT							3	
	Poly., -80°C		N/A		N/A		N/A	3	<0.0001
	Whatman							3	
28 days									
	Poly., RT	3		3		3		3	
	Poly., -80°C	3	0.3296	3	<0.0001	3	0.0128	3	0.0005
	Whatman	3		3		3		3	
56 days									
	Poly., RT	3		3		3			
	Poly., -80°C	3	0.0061	3	0.0188	3	0.0009		N/A
	Whatman	3		3		3			

[*] At $\alpha = 0.05$.

[a] P-values are for the comparison of the stated media types and storage conditions at the respective time point and concentration.

Kruskal-Wallis comparisons were also conducted for each media by enterococci cell concentration or effluent and accounting for all time points, excluding all samples with a Ct of 40 (Table VII). When pooling the Ct values of all concentrations (without effluent samples) and all storage lengths each media type and storage condition are statistically consistent with itself, with polycarbonate samples stored at -80^{o}C ($p = 0.8325$) performing more consistently than both polycarbonate samples stored at room temperature ($p = 0.6088$) and Whatman cards ($p = 0.5874$). For lower concentrations of 1×10^2 and 1×10^3 cells/mL there is a somewhat stark difference in reliability between the different media. Room temperature polycarbonate samples performed the most consistently at 1×10^2 cells/mL ($p = 0.4069$) while frozen polycarbonate samples performed similarly at 1×10^3 cells/mL ($p = 0.2168$), with respective other media types either weakly significantly or not at all consistent (Table VII). At 1×10^4 cells/mL both Whatman cards ($p = 0.2196$) and frozen polycarbonate samples ($p = 0.1979$) are much more consistent than room temperature polycarbonate samples ($p = 0.0046$). This might be expected based on previously noted trends of polycarbonate filter samples under both storage conditions experiencing an upward shift in Ct at longer storage times.

The same comparisons made for effluent samples, also excluding all samples with a Ct of 40, showed similar results to the combined concentration results. Polycarbonate samples stored at room temperature ($p = 0.8320$) performed most consistently, followed by Whatman card samples ($p = 0.3563$) and frozen polycarbonate samples ($p = 0.1079$) (Table VII). However, it should be noted that a far greater number of Whatman samples were excluded due to Ct value than polycarbonate samples at either storage condition, and that frozen polycarbonate samples included a single large outlier, which could potentially skew this comparison.

TABLE VII

KRUSKAL-WALLIS COMPARISON TEST RESULTS FOR MEDIA TYPE BY SAMPLE CONCENTRATION

Media Type (Storage Condition)	Kruskal-Wallis Statistic (p-value)				
	All Concentrations (no effluent)	**1 x 10^2 cells/mL**	**1 x 10^3 cells/mL**	**1 x 10^4 cells/mL**	**Effluent**
Whatman Card	0.5874	0.0888	0.0152	0.2196	0.3563
Polycarbonate (Room Temp.)	0.6088	0.4069	0.0131	0.0046	0.832
Polycarbonate (Frozen)	0.8325	0.0067	0.2168	0.1979	0.1079

* At α = 0.05.

4. DISCUSSION

The results of these research trials indicate that Whatman FTA cards can perform as possible alternatives to current methods of microbial DNA preservation for molecular analysis of surface water samples. While Whatman card median Ct values were consistently higher than those for polycarbonate filter samples under either storage condition (as expected, given that the number of cells applied to Whatman Cards was 1/1,000th of that applied to polycarbonate filters), they were highly consistent with each other by both subjective examination and according to Kruskal-Wallis test results, particularly at concentrations of 1 x 10^4 cells/mL and above (Table VII).

When grouped by cell concentration Whatman cards returned overall consistent enterococci Ct values, similar to polycarbonate samples stored at room temperature, but were slightly less consistent than frozen polycarbonate samples as determined by Kruskal-Wallis results. Whatman cards also have consistent enterococci Ct values across all storage lengths. When comparing all media types and storage conditions across time we can see a general trend of Ct values becoming more dissimilar as storage length increases; this is especially true for smaller concentrations (Table VI). In conjunction with analysis of sample medians it can be said that this trend may be due to polycarbonate sample Ct values trending higher with time, while Whatman Ct values remain relatively constant. This could indicate that Whatman cards perform better than polycarbonate filters for preserving microbial DNA for periods of up to 56 days.

When assessing wastewater effluent on Whatman card, samples displayed the same consistent Ct values over time, apparently more so than frozen polycarbonate samples. Small sample sizes as well as a high prevalence of nondetectable Ct values for Whatman cards have possibly skewed these results, however. During the extraction process Whatman samples may not be thoroughly saturated in lysis

buffer, leading to a less concentrated lysate for all sample types. Organic material and inorganic compounds found in wastewater effluent may also contribute to a less efficient extraction through physical or chemical interference with purification reagents. Along with the already comparatively dilute Whatman effluent samples any further loss of sample concentration could lead to artificially increased or inconsistent Ct values. A loss of efficiency in DNA recovery for Whatman cards could also contribute to a higher prevalence of nondetectable Ct values seen for effluent samples and samples with lower cell concentrations. Still, the trends observed in this limited data set indicate that Whatman cards perform similarly to polycarbonate filter samples when analyzing samples with unknown bacterial quantities. This is further reinforced when Whatman Ct values are adjusted to account for the 1,000-fold difference in sample volume when compared to the sample volume filtered through polycarbonate.

Overall, nonparametric comparison indicates that quantification of microbial DNA from Whatman cards produces Ct values that are consistent with one another at very low cell concentrations, remain consistent over long storage periods, and can likely be used for the detection of target species in samples containing mixed bacterial communities. Whatman cards perform similarly to, or slightly better than, polycarbonate filters in all such scenarios.

These results concur with previous studies evaluating the use of Whatman FTA cards in preserving microbial nucleic acid. Song et al. (2016) did not use qPCR in their study, but concluded that Whatman FTA cards are a viable method of preservation for bacterial DNA in animal fecal samples; similarly, Nechvatal et al. (2008) and Lalani et al. (2018) also had success preserving human fecal samples from clinical settings on Whatman cards for qPCR and DNA sequencing, with Lalani et al. storing some samples at ambient temperatures for up to 718 days; while our storage was limited to 56 days, we also observed consistent Whatman FTA Ct values across time. Other comparisons of long-term ambient

temperature storage methods include Rajendram et al. (2006), that stored 600 different bacterial strains on Whatman FTA cards for 3 years before successful quantification of DNA by PCR, with performance somewhat improved at concentrations of 1 x 10^3 cells/mL and above, a trend also observed in our data. A comparison of surface water collection methods from rivers in China (Worrell et al., 2011) used Whatman FTA cards to preserve and transport water samples from China to Atlanta, GA, USA, with qPCR results robust enough to draw significant conclusions between the two methods.

4.1 **Limitations**

While each trial attempted to control for as many factors as possible, there were some noteworthy inconsistencies. For those trials involving known quantities of *E. faecalis* cells, the stock cell aliquot used in the first two trials ran low and needed to be replaced with a new aliquot. While the new stock cell aliquot was diluted from the same master stock as the original aliquot, slight variances in initial dilution could impact the serial dilutions made later. New effluent samples were collected at the beginning of each trial from the same wastewater reclamation plant, which could also have varied in enterococci concentration and general composition. For this research, wastewater effluent was used as our environmental sample, though effluent typically has less suspended solids and more uniform composition than many surface water sources, meaning effluent provides less potential interference with DNA extraction or potential for PCR inhibition.

While all samples for each storage length and media were prepared concurrently, any incomplete mixing of effluent before filtration of each sample, improper handling of samples post-filtration, disruption of DNA extraction by organic materials accumulated on the filter, or another unrecognized deviation from protocol could all contribute to any observed abnormalities in the data.

A wider range of dilutions could have been carried from initial range finding trials through the subsequent storage trials to better describe the trends seen in Ct consistency for both media types. Sample size for each concentration, as well as for effluent for each storage condition, could have been increased. A smaller sample pool at later time points for each media type, especially when compared to the relatively even distribution of samples across concentrations, may lead to any outliers at these storage lengths having a greater influence in this instance. This specifically would have been important in the effluent trial, given the high prevalence of nondetect Ct values, and outliers with no immediate explanation. The limit of detection at 40 cycles for our assay producing a large number of nondetects made it difficult to see if a clear distinction existed for lower concentrations and effluent samples as well. Day 14 Ct distribution is especially susceptible to the influence of outliers as only effluent was analyzed at that storage length, with 3 samples were analyzed for each storage condition. A larger sample size would increase confidence in the results of the comparison tests. Repeated effluent storage trials would improve the overall robustness of the data set and perhaps allow for real-time adjustments to the Whatman card samples to have a more direct comparison with polycarbonate samples.

The heating apparatus used for incubation periods in Whatman card DNA extraction was changed from a water bath to a heat block after the first storage trial with known cell concentrations. Fluctuations in temperature between the two, or during the incubation periods, could affect the efficiency of the extraction and therefore skew the Ct results.

There were several instances of background contamination in NTC and MeB samples across all trials and sample types that may have also benefitted from the ability to repeat those trials, or from duplicate plates run concurrently, to establish whether the contamination was real or background.

4.2 **Conclusions**

Access to clean drinking water will likely remain a public health crisis for many years to come. Though current water quality methods are effective, sample collection and handling protocols can limit access to testing for more rural or low-resource areas. Based on this research, Whatman cards present an alternative method of microbial DNA preservation from surface water samples, with less limitations in sample handling and transport than current methods. These samples can be stored for at least two months at ambient temperatures without experiencing nucleic acid degradation, though Whatman cards produce more consistent Ct values at concentrations of 1×10^4 cells/mL and above.

The results of this research are supported by previous studies showing that Whatman cards are a viable method for preserving DNA for molecular analyses, compared to both cryopreservation and other ambient temperature storage methods, and added new evidence that Whatman cards can be used for the storage of environmental surface water samples. Still, further studies need to be done to confirm the place of Whatman cards in environmental sampling.

Drinking water typically has much lower concentrations of enterococci than 10^4 cells/mL, meaning that Whatman cards may not be useful for evaluating drinking water quality, though given the limitations of this study we can't say for certain. Trials need to be conducted on wide variety of environmental samples, including surface waters representing a spectrum of potential contamination levels and public health threat from a wide breadth of communities. Lab-made standards and effluent cannot be taken as direct representatives of the kind of surface water samples found in water-stressed communities; the most accurate way to describe Whatman card performance in these settings is to go into the field. Trials conducted in this study should also be repeated with larger sample sizes and greater consistency between sample volumes, cell concentrations, and storage lengths, to address some current

limitations. However, at this moment, this research would suggest that Whatman cards can be effective for public health applications like microbial source tracking and nucleic acid sequencing, which aren't reliant on quantification. These cards could assist in rapid response to disease outbreaks with precise, directed results, allowing those responders to alleviate as much potential suffering as possible.

This initial research makes us optimistic that Whatman FTA cards can be used to give researchers easier access to communities that were previously considered unreachable, or with limited water sampling and analysis resources. The ease of transport provided by Whatman cards creates more access to labs with molecular analysis capabilities, providing a more precise description of the unique microbial populations found in water-stressed communities. Understanding the source and type of microbial contamination can help guide public health efforts to build targeted water management and sanitation infrastructure to meet the needs of the community, and help to ease the global burden of waterborne diseases by bolstering access to safe drinking to those that need it most.

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APPENDICES

APPENDIX A

Recovery and Range Finding Trial Protocol

Objective: Following the previous limit of detection trial, we are attempting to establish a more precise limit of detection for Whatman cards, and any extra sample preparation steps compared to current SOPs that may be necessary to bring Whatman card LODs up to match that seen with polycarbonate filters.

Sample Preparation

1. Wash hands with soap and water. While wearing nitrile gloves, clean bench with bleach (allow to stand for 5 min) and 70% ETOH.
2. Remove calibration cells from freezer and allow to thaw completely. Vortex thoroughly (30 seconds minimum) before use.
3. Using calibration cells (concentration: 10^6 cells/mL, prepared 5/20/19), prepare serial dilutions of each of the following standards.
    - 100,000 (10^5) cells/mL
    - 10,000 (10^4) cells/mL
    - 1,000 (10^3) cells/mL
    - 100 (10^2) cells/mL
    - 10 (10^1) cells/mL
4. Label and prepare the following:
    - 16 1.5mL nuclease-free beaded extraction tubes
    - 16 1.5mL nuclease-free microcentrifuge tubes
    - 5 Whatman cards
5. Prepare 4 sterile filter housings with 47mm, 0.4μm polycarbonate filters [see EPA Method 1609.1].
6. Filter 30mL of sterile PBS as a method blank. Transfer filter to individual nuclease-free 1.5mL pre-filled bead tube.
7. Thoroughly vortex prepared stock of each standard. Filter 1mL of each standard with 99mL of sterile DI water, washing filter with PBS once entire sample has been pulled through the filter and no liquid remains. Transfer each filter to an individual nuclease-free 1.5mL pre-filled bead tube. Store at 4^{o}C until ready for use.
8. Pipette 100µL of sterile PBS onto one Whatman card sample area as a method blank.
9. Pipette 100µL of appropriate standard to reach desired final concentration onto Whatman card per 1-inch sample area (x3 for each standard). Allow each Whatman card to air dry at room temperature for 25 - 30 minutes or until completely dry. Store cards in a plastic bag with silica desiccator at room temperature until ready for use.

APPENDIX A (continued)

Storage Trial Protocol

Objective: Describe how storage conditions affect the integrity of bacterial DNA in Whatman cards (storage temp, storage length) vs. current polycarbonate filter storage/archiving methods.

Sample Set: This trial will comprise 6 "sample sets". All samples are prepped on the same day from the same stock cell dilutions, then filtered/applied to Whatman cards.

- 9 samples, 10^4 cells/mL (3 Whatman, 3 polycarb. stored at RT, 3 polycarb. stored at -80C)
- 9 samples, 10^3 cells/mL (3 Whatman, 3 polycarb. stored at RT, 3 polycarb. stored at -80C)
- 9 samples, 10^2 cells/mL (3 Whatman, 3 polycarb. stored at RT, 3 polycarb. stored at -80C)
- 9 samples, 10^1 cells/mL (3 Whatman, 3 polycarb. stored at RT, 3 polycarb. stored at -80C)
- 2 method blanks (1 for filters, 1 for Whatman)

1. Wash hands with soap and water. While wearing nitrile gloves, clean bench with bleach (allow to stand for 5 min) and 70% ETOH.
2. Remove calibration cells from freezer and allow to thaw completely. Vortex thoroughly (30 seconds minimum) before use.
3. Materials needed:
    - 125 1.5mL nuclease-free beaded extraction tubes
    - 13 1.5mL nuclease-free microcentrifuge tubes
    - 20 Whatman cards
4. Using calibration cells (concentration: 10^6 cfu/mL, prepared 5/20/19), prepare the following volumes of each of the following standards by serial dilution.
    - 10,000 (10^4) cells/mL
    - 1,000 (10^3) cells/mL
    - 100 (10^2) cells/mL
    - 10 (10^1) cells/mL
5. Prepare 4 sterile filter housings with 47mm, 0.4μm polycarbonate filters [see EPA Method 1609.1].
6. Filter 30mL of sterile PBS as a method blank, x6. Transfer filter to individual nuclease-free 1.5mL pre-filled bead tube.
7. Thoroughly vortex prepared stock of each standard. Apply 1mL of each standard to filter, then top off with 99mL sterile DI water. Filter x6 per sample set (total 36 per standard), washing filter with PBS once entire sample has been pulled through the filter and no liquid remains. Transfer filters to individual nuclease-free 1.5mL beaded extraction tubes.
    - For every sample set, store 3x each dilution at RT and 3x each dilution at -80C until ready for extraction.
    - For Sample Set 1, put aside <u>3x each dilution</u> (no -80^{o}C storage set) for immediate extraction.
8. Pipette 100µL of sterile PBS per 1-inch sample area x6 as method blanks.
9. Pipette 100µL of each sample onto Whatman card per 1-inch sample area (x3 for each standard). Allow each Whatman card to air dry at room temperature for 20 - 30 minutes or until completely dry.
    - For every sample set, store Whatman cards in plastic bags with dessicator at RT until ready for extraction.
    - For Sample Set 1, prepare Whatman samples for immediate extraction.
10. Sampling schedule: immediate extraction (0 days), 1 day, 28 days, 56 days

APPENDIX A (continued)

Effluent Trial Protocol

Objective: Evaluate if there is a difference in the recovery of enterococci between effluent samples filtered and stored on polycarbonate and effluent samples applied to and stored on Whatman FTA cards?

- When samples are quantified via qPCR we would expect to see consistent recovery for each media type across each storage length and temperature, with Whatman samples performing on par with polycarbonate samples stored at -80^{o}C.

Trial Setup

1. Wash hands with soap and water and put on nitrile gloves.
2. Spray benchtop with bleach and allow to sit for 5 minutes. Wipe up bleach with paper towels, then spray with 70% ETOH. Wipe up immediately.
3. Following sterile technique where possible, assemble vacuum filter apparatus with filter housings.
4. For a method blank, filter 30mL of 1X sterile PBS 9 times. Transfer each filter to an individual nuclease-free bead tube.
5. Filter 1mL of 10^6 cells/mL calibrator cells 5 times. Transfer each filter to an individual nuclease-free bead tube.
6. Gather wastewater effluent samples. Filter 100mL of effluent x27 onto polycarbonate filters. Transfer filters to sterile nuclease-free bead tubes. Store 12 filter samples plus 4 each of MeB/CAL at -80^{o}C, and 12 filter samples plus 4 MeB at RT until ready for use. Reserve the last 3 filter samples and MeB/CAL for immediate extraction.
7. Label two Whatman cards as method blanks. While holding the inner sample card up off of the card backing, slowly pipette 100µL of 1X sterile PBS into the center of one sample area as a method blank. Repeat 4 times, for a total of 5 method blanks.
8. Label 10 Whatman cards as effluent sample with extraction date. For 5 Whatman cards, pipette 100µL of effluent sample into the center of each of the 4 sample areas while holding the inner sample card up off of the card backing. For the other 5 Whatman cards, pipette 100µL of effluent sample into 2 sample areas. Set aside a total of 6 Whatman samples (1 card with 4 samples, 1 card with 2 samples) for <u>each</u> extraction date.
9. Prop Whatman cards up and allow to air dry for a minimum of 20 minutes. Store in a plastic bag with silica desiccator at room temperature until ready for use.

APPENDIX A (continued)

DNA Extraction - Whatman Card (Qiagen [QIAamp DNA Investigator Kit] method)

1. Using a sterilized razor blade, cut out each 1 inch sample disk from Whatman card and transfer to labeled nuclease-free 1.5mL microcentrifuge tube.
2. To each tube, add 280µL ATL buffer and 20µL of ProK.
3. Vortex each sample for a minimum of 30 seconds.
4. Heat for 60 minutes at 56◦C, vortexing each sample for 10 seconds every 10 minutes throughout the incubation.
5. Centrifuge at 12,000 x g for 30 seconds.
6. Add 300µL AL buffer and vortex each sample for 10 seconds.
7. Heat at 70◦C for 15 minutes, vortexing each sample for 10 seconds every 3 minutes throughout the incubation.
8. Centrifuge at 12,000 x g for 30 seconds.
9. Transfer 600µL eluate (entire contents of each tube) to QIAprep spin column.
10. Centrifuge at 8,000 x g for 1 minute. Discard flow through.
11. Add 700µL AW2 buffer to spin column.
12. Centrifuge at 8,000 x g for 1 minute. Discard flow through.
13. Add 700µL of 100% ETOH to spin column and centrifuge at 8,000 x g for 1 minute. Discard flow through.
14. Spin at 12,000 x g for 4 minutes.
15. Transfer spin columns to labeled, nuclease free 1.5mL microcentrifuge tubes. Open spin column lid and allow ETOH to evaporate for 10 minutes on the benchtop.
16. Add 25µL of sterile PCR-grade water to spin column and allow to incubate at room temperature for 5 minutes.
17. Spin at 12,000 x g for 1 minute. Discard spin columns. Keep tube with eluate. Store at 4◦C until ready for use.

APPENDIX B

TABLE VIII
SUMMARY OF CITED LITERATURE REVIEW STUDIES

Author (Year)	Sample Type	Target	Type of Card	Preservation Methods	Results
Rajendram, D. et al. (2006)	Bacteria, lab-cultured	600 various bacterial strains.	Whatman FTA cards	Whatman cards were stored for 3 years at room temperature; 100 strains randomly selected for analysis.	No viable cells retrieved from gram-negative bacterial species. Some gram-positive species were viable at concentrations of 10^4 cfu/mL and above. Of the 100 randomly-selected strains preserved for 3 years prior to PCR analysis, all produced consistent intensity (looking for 16S RNA fragment via gel electrophoresis), and DNA sequencing of each sample produced proper identification of each strain.
Song, S.J. et al. (2016)	Fecal samples, human and dog	Various fecal bacterial targets.	Whatman FTA cards	Whatman cards stored at room temperature for up to 8 weeks.	Analyses used in this study not as sensitive as qPCR, though Whatman FTA cards are recommended as a potential long-term storage method for microbial DNA.
da Cunha Santos, G. (2018)	Cytologic/tissue samples	Nucleic acid, immunoglobulins, various cancer markers.	Whatman FTA cards	Various, but some samples stored on Whatman cards at ambient temperature up to 2 years.	Quality nucleic acid could be retrieved from Whatman FTA cards to accurately identified cancer markers, HIV, immunoglobulins, and other targets. Results were comparable to the quality of nucleic acid stored via cryopreservation.
Nechvatal, J.M. et al. (2008)	Fecal samples, human	Various fecal bacterial targets.	Whatman FTA cards	Fecal samples spread and dried on Whatman FTA cards and stored at ambient temperature for 5 days.	DNA targets were successfully preserved and extracted from all preservation methods, including Whatman FTA cards, dried over silica gel beads, submersion in 1.0 ml RNAlater, immersion in 1.0 ml Paxgene, and refrigerator storage.
Lalani, T. et al. (2018)	Fecal samples, human	Fecal bacteria associated with travelers' diarrhea.	Whatman FTA Elute cards	Combined median storage and ship time was 712 days at ambient temperature.	Not much of a difference between recovery from FTA card and frozen stool samples, though FTA performed slightly better with higher peaks
Gray, M.A. et al. (2012)	Bacteria, lab-cultured	Mixed microbial communities representative of a marine surface water environment.	Whatman FTA cards, Whatman FTA Elute cards	Whatman cards stored for 1 week, 1 month, 3 months, and 6 months at RT away from light.	Liquid DNA preservation methods (DNAgard, RNAlater, DMSO-EDTA-salt (DESS)) outperformed both Whatman cards; Whatman cards showed less successful preservation of gram+ bacterial DNA.
Worrel, C. et al. (2011)	Environmental surface water	*S. japonicum* cercariae (free-swimming larval stage of parasitic lifecycle) from surface freshwater samples.	Whatman FTA cards	Stored on card at ambient temp and shipped from China to Atlanta, GA, USA.	DNA retrieved from Whatman cards for analysis performed as expected via mouse bioassay (standard detection protocol), with comparatively improved performance using qPCR.

APPENDIX B (continued)

TABLE IX
INITIAL KRUSKAL WALLIS COMPARISON TEST RESULTS BY MEDIA TYPE AND CONCENTRATION

By Storage Condition

All media types and storage conditions, no effluent samples

	n	p-value
Poly., Room Temperature	126	<0.0001
Poly., Frozen -80°C	72	
Whatman Card	126	

All media types and storage conditions, effluent samples

	n	p-value
Poly., Room Temperature	33	<0.0001
Poly., Frozen -80°C	21	
Whatman Card	26	

Direct Comparison of poly. RT and Whatman

	n	p-value
Poly., Room Temperature	159	<0.0001
Whatman Card	152	

Direct Comparison of poly. -80°C and Whatman

	n	p-value
Poly., Frozen -80°C	93	<0.0001
Whatman Card	152	

By Sample Type

All storage conditions and media types, no effluent samples

	n	p-value
Polycarbonate, 10^4 cells/mL	51	
Whatman Card, 10^4 cells/mL	33	
Polycarbonate, 10^3 cells/mL	51	<0.0001
Whatman Card, 10^3 cells/mL	33	
Polycarbonate, 10^2 cells/mL	48	
Whatman Card, 10^2 cells/mL	30	

Direct Comparison of media performance at 10^4 cells/mL(a)

	n	p-value
Polycarbonate, 10^4 cells/mL	51	<0.0001
Whatman Card, 10^4 cells/mL	33	

Direct Comparison of media performance at 10^3 cells/mL(a)

	n	p-value
Polycarbonate, 10^3 cells/mL	51	0.0345
Whatman Card, 10^3 cells/mL	33	

Direct Comparison of media performance at 10^2 cells/mL(a)

	n	p-value
Polycarbonate, 10^2 cells/mL	48	<0.0001
Whatman Card, 10^2 cells/mL	30	

* All cell concentrations ranged between 1 x 10^2 and 1 x 10^4 cells/mL for this analysis.

[a] All storage conditions (room temperature and -80°C) were combined for polycarbonate.

APPENDIX B (continued)

TABLE X
INITIAL KRUSKAL WALLIS COMPARISON TEST RESULTS BY TIME AND CONCENTRATION

Summary of all storage lengths, all media types and concentrations grouped

Storage Length	n	p-value
Immediate	132	0.109
1 day	101	
7 days	81	
14 days	9	
28 days	45	
56 days	36	

Comparison of Enterococci Ct across storage time, media and storage condition

Sample Concentration	Storage Length	Poly., Room Temperature, n	Poly., -80°C, n	Whatman, n	p-value
1×10^2 cells/mL	Immediate	12	N/A	12	0.0139
	1 day	6	6	6	
	7 days	6	6	6	
	28 days	3	3	3	
	56 days	3	3	3	
1×10^3 cells/mL	Immediate	15	N/A	15	<0.0001
	1 day	6	6	6	
	7 days	6	6	6	
	28 days	3	3	3	
	56 days	3	3	3	
1×10^4 cells/mL	Immediate	15	N/A	15	<0.0001
	1 day	6	6	6	
	7 days	6	6	6	
	28 days	3	3	3	
	56 days	3	3	3	
Effluent	Immediate	15	N/A	9	<0.0001
	1 day	9	12	8	
	7 days	3	3	3	
	14 days	3	3	3	
	28 days	3	3	3	

APPENDIX B (continued)

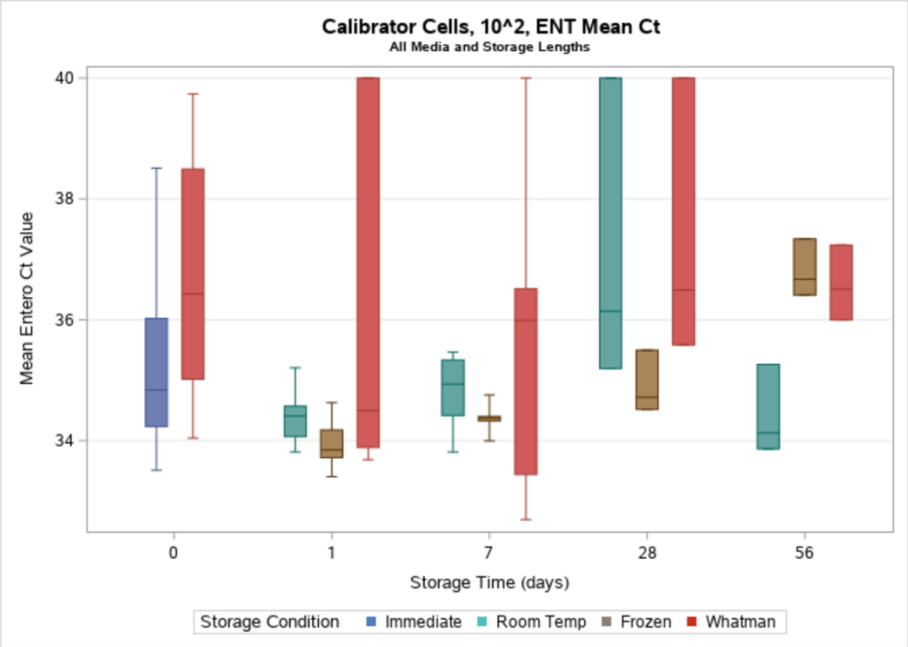

Figure 6. Visualization of the distribution of enterococci Ct values for samples at a concentration of 1 x 10^2 cells/mL, grouped by storage length. Whiskers represent 5^{th} and 95^{th} percentiles. Hash mark within each box represents the median.

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