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Testing the potential for field qPCR detection of fecal indicators in the Saw Kill, a Hudson River tributary

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by

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Every accomplishment from my time at Bard College is dedicated to all of my loved ones who supported me throughout my college years. To my parents, Erika and Mauricio Medina, for your endless love and encouragement–there are no words to fully express my gratitude. To my brothers, Warachi and Zoshio. To Grandpa Bob and Nana, thank you for your support with my schooling. And thank you to everyone else around me who cheered me on these past several years.

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Abstract

Water quality is a prominent issue across the globe, with pathogenic bacteria being the leading cause for many illnesses and infections. Although testing for these bacteria can be difficult, there are some specific bacteria, known as fecal indicator bacteria (FIB), which are easier to test for. Indicating the presence of untreated waste, these bacteria are an important aspect to monitoring water quality. Current methods for monitoring FIBs rely primarily on the use of culture based methods. These methods require a 24-hour incubation, however, and also do not detect antibiotic resistant bacteria and micro pollutants present in treated and untreated sewage releases. As our reliance on antibiotics for health issues and the use of antibiotics in agriculture increases, we are seeing an alarming increase in antibiotic resistant bacteria. The Int1 gene, capable of transferring the antibiotic resistance between and among bacteria is an important indicator that should be tracked. The Biomeme Franklin® Real-Time PCR Thermocycler is a new piece of technology designed to test for the presence of fecal indicators such as *E. Coli* and Total Coliforms, and has been suggested as a new way to monitor sewage pollution in freshwater systems. With a shorter run-time than culture-based methods, this new technology is showing potential for being applicable to testing for genes like In1. In this study, I tested for this potential by first determining and optimizing a protocol for the use of the Biomeme on past samples of the Saw Kill, a Hudson River Tributary, and then comparing it to FIB measurements. Although the Biomeme is able to detect the presence of *E. Coli* and Human Fecal associated bacteria, it did not correlate with culture-based methods. Before the Biomeme's capacity to detect Int1 can be evaluated, these inconsistencies between qPCR and culture-based methods must first be further researched.

Introduction

Streaming down from its headwaters in Milan NY, the Saw Kill, named Metambesem by the Stockbridge-Munsee Band of Mohican people, is a tributary of the Hudson River. The Hudson River was originally named Mahicannituck which translates to the river that flows both ways. Gaining this name because of the effects of the tides that push and pull the river, the flow changes even as far inland as the Tivoli Bays where the Saw Kill meets the Hudson. While the river itself is 14.3 miles long the Saw Kill watershed is an area encompassing about 26.2 square miles, with the water running through various towns and farms until it reaches the Hudson River ("Saw Kill Watershed," n.d.). In the nineteenth century, during the Hudson River School of Art movement the Saw Kill was featured in the art of architect Alexander Jackson Davis and artist Jacques-Gerard Milbert. In 1841 the conservation covenant, formed to protect the Saw Kill, allowed for the river to flourish, even as logging, agriculture, and milling industries expanded throughout the Hudson Valley ("The History of the Saw Kill" 2015). Home to a diverse variety of aquatic life including Species of Greatest Conservation Need and Special Concern within New York State, such as the American Eel (*Anguilla rostrata*), the Louisiana Waterthrush (*motacilla*), and the Wood Turtle (*Glyptemys insculpta*), (Kiviat, 2021) the Saw Kill plays an important role in the culture and health of local ecosystems, human and non-human alike.

There are several towns and villages in the Saw Kill watershed, including Red Hook, Milan, Tivoli, and Rhinebeck. The homes and businesses in these areas use the Saw Kill for their drinking water and rely on it as a wastewater discharge site. Bard College is also within the watershed, drawing their water for the student body of approximately 1,800 undergraduates and 270 faculty just upstream of their effluent discharge pipe ("Food, Water, + Land Use," n.d.). In addition there are several farms throughout the area that rely on the Saw Kill. The close

proximity of these farms to the river often results in farm animal waste and agricultural run-off into the Saw Kill water system ("US EPA. Ag_runoff_fact_sheet.Pdf," n.d.; OW US EPA 2015). Between farms, wastewater treatment plants (WWTPs), and general human infrastructure there is significant human-associated bacteria being introduced into the Saw Kill watershed, altering pre-existing microbial communities in the environment (De Santana et al. 2022). Some of these bacteria are naturally occurring such as *Esherichia coli* (*E. coli*) and *Enterococcus* which are present in the human and animal gut. Expelled through waste, exposure to these bacteria through drinking or swimming in contaminated water or eating contaminated food can cause mild to severe illness, usually presenting as gastroenteritis and diarrhea. According to the World Health Organization (WHO), there are an estimated 485,00 deaths and 111 million illnesses annually linked to diarrheal diseases ("Escherichia Coli, Diarrheagenic | CDC Yellow Book 2024," n.d.; Oon et al. 2023).

There are two primary ways that these bacteria enter the environment: through point source and nonpoint source pollution sites. Point source is largely from wastewater treatment plants, whereas nonpoint source comes from wildlife, leakage from septic tanks, and agricultural runoff, which is much harder to track (Garcia-Armisen and Servais 2007). Bacteria associated with wild animal waste are always going to be present in low levels as they occur naturally, however it is important to monitor the concentration levels as these bacteria not only pose health risks, but can also be an indication of untreated waste from septic tanks and wastewater treatment plants (Stec et al. 2022). Untreated waste in our water systems is a cause for concern as waste holds a variety of bacteria and viruses and exposure can lead to serious health issues. However, as most of the different microbes require distinct testing, doing so for multiple pathogens is a

lengthy and difficult process which becomes impractical for frequent monitoring ("US EPA. 2009_03_13_estuaries_monitor_chap17.Pdf," n.d.).

Although testing for various pathogens can be difficult, there are some specific bacteria that are easier to test for. These bacteria, known as Fecal Indicator Bacteria (FIB) are monitored as indicators of untreated waste in water, both human and animal (Motlagh and Yang 2019). Bacteria such as *E. Coli, Enterococcus*, and Total Coliforms are all monitored through culture-based methods such as the IDEXX. In short, aftering taking a sample of water and mixing it with a growth medium, the sample is then incubated, allowing for bacteria colonies to form. After a 24 hour incubation period, the growth medium, once eaten by the bacteria, gives off a fluorescence, allowing the bacteria colonies to be counted by the naked eye or through the use of a blue light (Oon et al. 2023). The Environmental Protection Agency (EPA) set various standards and limits on the presence of pollutants in drinking water and water for recreational use, as well as supporting municipal wastewater treatment plants (OMS US EPA 2013). Based on their criteria, the single sample safety threshold of *E. Coli* for recreational water use is 235 Colony Forming Units (CFU) per 100 ml and 400 CFU per 100 ml for Total Coliforms. These regulations are a part of the Clean Water Act, which regulates quality standards and the presence of pollutants in surface waters ("US EPA. Ca4-Update-Bacteria.Pdf," n.d.). Culture-based methods have been the primary way of monitoring FIB concentrations for recreational water use for decades (Converse et al. 2012). However because of the nature of the incubation process and the materials required for prepping the IDEXX samples, there are limitations to the IDEXX turn around time and in-field capabilities. Culture-based methods are also unable to detect micro pollutants, including antibiotic resistant bacteria(ARG), present in treated and untreated sewage release (Ahmed et al. 2021).

Among the countless benign or beneficial bacteria we are exposed to on a daily basis, there are many that can cause serious infection and illness ("Bacteria: The Good, the Bad, and the Ugly" 2013). Thankfully, antibiotics were developed to address this. When the first antibiotics were discovered in the early nineteen-hundreds, there was an incredible shift in modern medicine. Antibiotics helped to save countless lives, and lessened the risk for surgeries and medical procedures (Ventola 2015). However, not long after the first antibiotics came the realization of antibiotic resistant bacteria. Unfortunately, given their short life cycles when exposed to an antibiotic, bacteria are quick to build resistance to it, with this "information" held in its genes. Because bacteria are able to acquire genes through mobile genetic material, this horizontal gene transfer allows antibiotic resistance to be transferred between various species of bacteria (Kaplan, 2014.). Modern medicine relies heavily on the use of antibiotics, sometimes excessively so, for human health in addition to pets and livestock (Ventola 2015). Data collected from varying sources indicates the use of antibiotics is steadily increasing worldwide at a rate of 30% (Mutuku, Gazdag, and Melegh 2022). The extensive use of low-dose antibiotics in livestock feeds has become a common global practice due to the benefit of the boost in animal and bird growth it provides. Each year, approximately 24.6 million pounds of antibiotics are used in livestock farming (Mutuku, Gazdag, and Melegh 2022), linking the overuse of antibiotics in animal production as a leading source of antibiotics in the environment. These pharmaceutical contaminants have even been found to be present in polar regions, the most pristine environment on earth (González-Alonso et al. 2017). This global unprecedented increase in the use of antibiotics is producing an increase in resistant bacteria. Antibiotic overprescribing by primary care physicians attempting to treat viral infections is also a pervasive problem. Studies show that about 90% of all antibiotic prescriptions are issued by general practitioners, with respiratory tract

infections as the leading reason for prescribing (Llor and Bjerrum 2014). Antibiotics are only useful as long as they are capable of fighting off pathogenic bacteria and this overprescribing is contributing to a growth in bacterial resistance, causing a decline in the effectiveness of pharmaceutical antimicrobials which is in turn causing higher medical costs and mortality rates (Mutuku, Gazdag, and Melegh 2022). Hospital effluence, animal farms, aquaculture and municipal waste are all sources for the emergence of ARGs into the aquatic environment, mainly through agriculture run-off and the flow of WWTP effluent into surface or groundwater (Mutuku, Gazdag, and Melegh 2022).

The process of wastewater treatment plants vary, but the methods are largely similar. There is a primary treatment, where large debris and grit such as rocks or stones are filtered and removed. The waste then enters the sedimentation tank, where suspended solids will sink to the bottom and be disposed of. The effluent then moves to secondary treatment, which involves making use of bacteria to consume remaining organic matter. Once this process is complete, the waste undergoes a chlorination process, designed to kill pathogenic bacteria, before the wastewater is dechlorinated and released ("Bastre.Pdf," 1998.). What is troubling is that even when a bacteria is "killed" through the wastewater treatment process it retains the genetic information for antibiotic resistance, making it possible for this information to be captured and taken up by living bacteria, allowing these living bacteria to build antibiotic resistance without ever having been directly exposed themselves (von Wintersdorff et al. 2016). Specifically, this is possible through the class 1 integron-integrase (Int1) gene. Int1 is a mobile genetic element (MGE) capable of capturing and expressing ARGs (Keenum et al. 2022) which are often found in WWTPs. As our use of antibiotics increases, antibiotic resistance is becoming more abundant within our environment (Zheng et al. 2020). As Int1 is a mobile genetic element capable of

transferring these genes between bacteria, when antibiotic resistance is thought to be present it is important to track this gene, especially as much is still unknown regarding its accuracy for monitoring antibiotic resistance (Zheng et al. 2020). Indicative of pollution, this gene can potentially be a reliable aid in monitoring ARGs. At this time, one of the most reliable methods for monitoring is by the qPCR (Böckelmann et al. 2009). Quantitative polymerase chain reaction (qPCR) is essentially a process for turning a small amount of DNA into a significant amount, allowing for characteristics of the DNA to be read (Staff 2020).

Although Int1 is not yet used as an indicator, there are other indicators used to ensure water quality (Tyagi et al. 2020). Given Bard College's close proximity and reliance on the Saw Kill, there has been a water quality monitoring program in place since 2015, restarted after it initially began in 1976 where it ran until 1982. Once a month from February through December, several sites along the Saw Kill are tested using various water quality parameters ("StateOfTheSawKill-Overview.Pdf," n.d.). On location the river is tested for temperature, dissolved oxygen, conductivity, and pH. Water is collected in acid washed and autoclave sterilized 2L bottles, and brought back to the lab for further analysis. Once in the lab, samples are taken and run through the IDEXX to measure the abundance of *Enterococcus, E. Coli*, and Total Coliforms. The water is also measured for colored dissolved organic matter (CDOM), chlorophyll a, phycocyanin, and turbidity. 750 mL water samples from each site are run through 0.22 μm Sterivex filters, which are then frozen for DNA extraction and analysis (De Santana et al. 2022). Monitoring water quality indicators and measuring fecal bacteria via culture-based methods are important, however this still does not address the need for monitoring the Int1 gene. Recently, in hopes of addressing this gap in monitoring, there has been a new piece of technology that has been suggested as a new way to monitor sewage pollution in freshwater

systems (Li et al. 2020). The Biomeme Franklin® Real-Time PCR Thermocycler is a new piece of technology designed to test for the presence of fecal indicators such as *E. Coli, Enterococcus*, and Total Coliforms. Capable of being used in the field and with a shorter run-time than culture-based methods, this new technology is showing potential for being applicable to testing for pollutant indicators such as Int1 (Li et al. 2020). Currently, the Biomeme is being used to test for the presence of FIBs. When the sample is added to the BioPoo® E. Coli RT-PCR Go Strips®, which are pre-aliquoted lyophilized strips containing a mix of enzymes and multiplexed primers and probes, the reaction is read by the Biomeme. It then determines the presence of *E. Coli, Enterococcus,* and Total Coliforms (Fernández-Baca et al. 2021). Because of the ease in using the Biomeme without the requirement of a large incubator or incubation period, it has the potential to be much more accessible for community science then other technologies currently available. Additionally, it could potentially be used to fill the gaps in monitoring other micropollutants and indicators.

My senior project is focused on the preliminary research that needs to happen to determine whether or not the Biomeme will be useful, not only for FIBs, but for other genetic indicators including Int1. To do so I will be focusing on the Saw Kill, using preserved Sterivex filters from historical sampling. Because the Saw Kill is relatively small with detailed mapping of the location of effluent pipes from towns and communities such as Red Hook and Bard College, it can be easier to narrow down possible sources of contamination. Additionally, the Saw Kill Monitoring Program (SKMP) has been collecting monthly data for nearly 10 years. This not only gives us over a decade of information to study to determine how water quality has changed over the years, but it also means we have access to frozen Sterivex filters starting from 2015. In using these frozen samples I hope to address gaps in our understanding of how the

Biomeme could apply to various pollutants and indicators, including the Int1 gene. Culture-based methods are able to accurately determine various health risks and can be implemented with relative ease, making them the testing method of choice in many situations. However, results are not immediate and do not allow for the monitoring of all micro pollutants (Converse et al. 2012). It is my hope that with study will show the Biomeme's capabilities in filling some of these monitoring gaps. Although culture-based methods such as the IDEXX work very differently then qPCR methods there should still be some correlation between the Biomeme results and the IDEXX results. If the Biomeme is capable of detecting indicators accurately, the test results should correlate to IDEXX readings of *E. Coli* and Total Coliforms.

Methods

Sample sites and collection

Water samples were collected using methods based off of De Santana et al. In brief, the study site is located on the Saw Kill, a tributary of the Hudson River. Samples collected for this project were taken starting with Site 1, located on Bard College campus just as the Saw Kill reaches Tivoli South Bay. Based on the Saw Kill Monitoring Project sites shown in Figure 1, the sample sites are located primarily on Bard College campus and in the town of Red Hook.

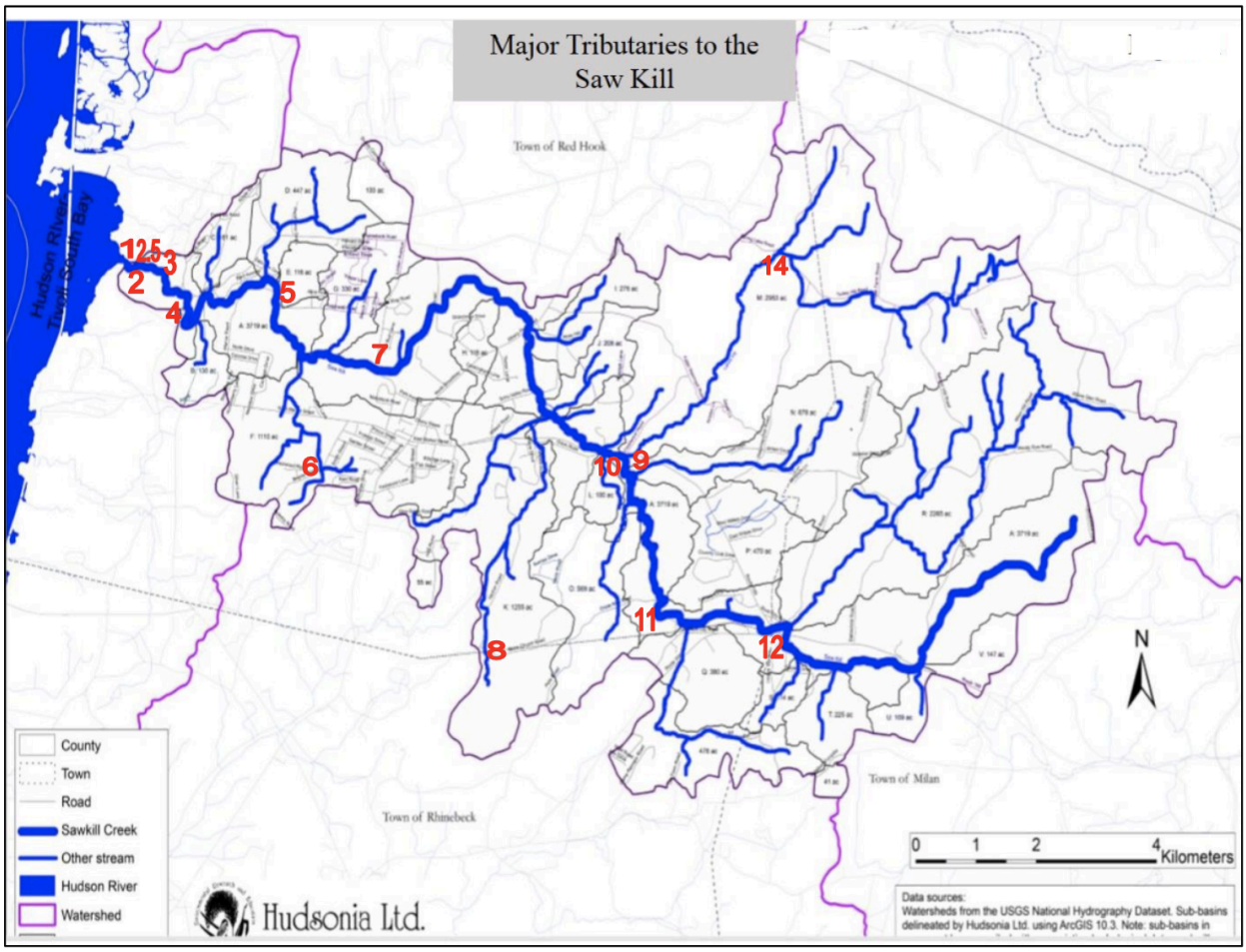

Figure 1. The Saw Kill and its major tributaries. Sampling sites are numbered and shown at their location. Most of the sampling sites are located on Bard College campus and in the town of Red Hook.

Bard College, located in Annandale-On-Hudson, relies on the Saw Kill as its primary source for drinking water, as well as disposing of its wastewater downstream of the drinking water intake. Bard's wastewater treatment plant utilizes a multi-step system, first going through filtration, sedimentation, fermentation in a bioreactor network, and then chlorination. Afterwards the treated wastewater experiences aeration and is de-chlorinated before being released into the Saw Kill through an outflow pipe. Using the sample methods outlined in De Santana et al., samples were taken once a month during April and May of 2019. To avoid possible contamination that could occur from disturbing the stream sediment, samples were collected

downstream first, moving upstream as they went. At each site, before collecting water samples, water temperature (ºC), conductivity (µmhos/cm), turbidity and dissolved oxygen (ppm) were measured using handheld YSI probes. The pH was also measured using an Oackton PCTSTester. To ensure accuracy these probes were calibrated before being taken into the field. Using acid washed and autoclave sterilized 2 liter bottles, water was collected from the sample sites. For sites accessed by a bridge, buckets were lowered, rinsed three to fives times in the stream prior to collection to lessen the risk of contamination, and then filled and pulled up to pour into the waiting bottle. Between sites bottles were kept in coolers with ice packs to slow microbial growth and activity.

Once back in the lab, the samples were once again handled following methods outlined in de Santana et al. In brief, using EPA-approved standard methods for IDEXX MPN measurements, *E. Coli, Enterococcus* and Total Coliforms samples were prepared. To prepare for measuring *Enterococcus*, an undiluted 100 mL of sample was added to a sterile 100 mL bottle and mixed with the IDEXX Enterolert growth medium. The process for *E. Coli* and Total Coliforms is similar to that for *Enterococcus*, however the water sample is diluted 1:10 and mixed with an IDEXX Colilert reagent. After the reagent dissolves completely both the Enterolert mixture and the Colilert mixture are poured into 49-well sterile Quanti-Trays and set to incubate for 24 hours, the Colilert at 35ºC and the Enterolert at 41ºC. After incubation, all cells in the Coliert trays that have turned yellow are considered positive and counted for coliform, while all yellow cells that fluoresce under UV lights are considered positive for *E. Coli*. Following incubation for the Enterolert trays, all cells that fluoresce are considered positive for *Enterococcus*. The positive cells are then counted and calculated at MPN/100 mL by

applying the Most Probable Number method (MPN) ("IDEXX Quanti-Tray*/2000 MPN Table," n.d.).

In addition to the IDEXX measurements, each site sample is measured in the lab for turbidity, colored dissolved organic matter (CDOM), chlorophyll a, and phycocyanin. 750 mL of water samples are then filtered through a 0.22 μm Sterivex filter. These filters are frozen until ready for use to extract DNA from, following the manufacturer's instructions for the Powerwater Sterivex Kit.

SKMP Sterivex DNA Extractions Protocol (Powerwater Sterivex Kit)

DNA was extracted from the frozen Sterivex filters based on the manufacturer's instructions for the Powerwater Sterivex Kit. Preliminary steps began with solutions ST1A and ST1B which are mixed and stored in the fridge. Procedure began with adding 0.9 ml of solution ST1B to the inlet cap, which was then capped and secured horizontally to the vortex adapter on the vortex genie 2 and spun at minimum speed for 10 minutes. The Sterivex filter unit was rotated 180 degrees and vortexed once more at minimum speed for another 10 minutes. Afterwards 0.9 ml of Solution MBL was added using a pipette tip, before the Sterivex filter is incubated at 90º for 5 minutes. After cooling at room temperature for 2 minutes the filter was attached horizontally to the vortex adapter and vortexed at maximum speed for 10 minutes. Once this step was complete, the lysate was removed from the filter and added to 5 ml glass Power Bead Tubes, which was then vortexed at maximum speed for 10 minutes. Afterwards it was centrifuged at 4000 x g for 1 minute. The supernatant was then transferred to a clean 2.2 ml collection tub, which was mixed thoroughly with 300 μl of Solution IRS. It was then refrigerated for 5 minutes, while solution MR was warming in a 65ºC water bath. The tube was centrifuged again at 13,000 x g for 1

minute, after which the supernatant was then transferred to a clean 5 ml collection tube. 3 ml of Solution MR was mixed into the 5 ml collection tube containing supernatant. After placing a tube extender onto the MB Spin Column, the unit was attached to a port on the QIAvac 24 Plus Manifold. The entire 5 ml collection tube containing the supernatant was loaded onto the MB Spin Column. After turning on the vacuum source, the VacValve of the port was opened, the lysate was allowed to pass through completely, and the vacuum source was subsequently turned off. The MB Spin Column was kept attached to the manifold as the tube extender was removed and discarded. 0.8 ml of ethanol was added to the MB Spin Column, after which the VacValve was opened to allow the ethanol to pass through completely. After shaking Solution PW, 0.8 ml of the solution was added to the MB Spin Column. The VacValve was opened and the Solution PW passed through the column completely. The vacuum was allowed to pull for another minute to dry the membrane before the VacValve was closed once more. Another 0.8 ml was added to the MB Spin Column, as the vacuum was turned on to pull the ethanol through the MB Spin Column completely, after which the vacuum continued pulling for another minute to dry the membrane. The VacValve was then closed and the vacuum source was turned off. The MB Spin Column was removed and placed in a 2.2 ml collection tube which was then centrifuged at 13,000 x g for 2 minutes to ensure the membrane was completely dry. Following this the MB Spin Column was then transferred to a new 2.2 ml collection tube and 100 µl of Solution EB was added to the center of the white filter membrane; this is considered “step 36”. After labeling, the collection tube was incubated at room temperature for 10 minutes. It was then centrifuged at 13,000 x g for one minute, rotated 180º and centrifuged at 13,000 x g for another minute. At this point the DNA is now ready for downstream application. The final step is to prepare a second

elution starting with “step 36”, afterwhich the collection tubes were labeled appropriately, to distinguish the first elution from the second elution.

Biomeme Protocol

The Biomeme consists of the Biomeme itself, the BioPoo® E, Coli RT-PCR Go-Strips®, and their caps, as well as a phone with the Biomeme app pre-installed. Connected via Bluetooth to the phone and controlled through the app, the Biomeme stores all information within the app after a run is completed. The Go-Strips® are pre-aliquoted lyophilized strips already containing a mix of enzymes and multiplexed primers and probes to react with the sample. Sample water is pipetted into the Go-Strip, and then mixed with DNase/RNase free water to reach 20 µl. The sample is then mixed with the primers by gently pipetting up and down 5 times. All the run information is stored within the app, as well as it being the control center for the Biomeme. After connecting the app to the Biomeme the test settings are loaded by scanning the barcode on the Go-Strips® bag. The machine is started, with the run time being about 48 minutes. Afterwards, the numbers are displayed and the information can be read.

Results and Discussion

Biomeme Protocol Optimization

The Biomeme uses real-time PCR technology, meaning it offers immediate results as the test is running. Challenges arose when trying to determine the dilution required for running the samples in the Biomeme. According to the manufacturer's instructions, the Biomeme needs 20 µl of sample, however the DNA extraction process only leaves us with 100 µl for various tests still needed, including the Qubit Fluorometer, which is capable of quantifying RNA, DNA, and

protein from a sample. Using 20 µl of our sample for the Biomeme is simply not sustainable, so I set out to determine how small of a dilution can be used successfully. I began with three different sample volumes: 1 µl, 5 µl, and 10 µl of sample combined with DNase/RNase free water to reach 20 µl. I also ran it with the full 20 µl sample for comparison. The water samples used for these tests were taken from Saw Kill sample sites 1-3 during early February 2024 for the purpose of protocol optimization. This process can be seen in Table 1 and 2. After discovering that dilutions below 10 µl became increasingly inconsistent in their results, as can be seen in the tables below, it was decided that 10 µl would work best. However, once I moved to using the stored samples from past years, I realized I was running into further issues. Using frozen samples from April and May of 2019, I noticed that at 10 µl many of the Biomeme runs were not working, and needed to be frequently re-run. Alongside the results for *E. Coli* and Human Fecal, the Biomeme runs an Internal Positive Control (IPC). When the IPC reads as 0.00, it is indicating that the run failed to be properly read. When running historical Saw Kill samples from 2019 at 10 µl of sample mixed with 10 µl of DNase/RNase free water, these misread runs became frequent, indicating an issue. Initially unsure of what the problem may be, upon further research I discovered the possibility of this misread being due to natural inhibitors. There are various micropollutants within each water sample, and although the process for preparation should have eliminated many of these contaminants, it is still possible for some of those inhibitors to be interfering with the Biomeme's results ("BioPoo Assay on eDNA," n.d.). With this realization, I decided to change the dilution from 10 µl to 3 µl, hoping to lessen the presence of these inhibitors.

Table 1. Biomeme Protocol Optimization: determining the appropriate dilution for measuring Total Coliforms with the Biomeme, then comparing it with the IDEXX Total Coliforms. Labeling convention for extractions: type+sample site_month+day+year+rep (if any). To clarify, SK indicates a sample from the Saw Kill, and EX indicates it as being an extraction optimization sample.

Sample	Sample Date	IDEXX *E. Coli*	Biomeme *E. Coli* 1 µl	Biomeme *E. Coli* 5 µl	Biomeme *E. Coli* 10 µl	Biomeme *E. Coli* 20 µl
EXSK1_020624A	2/6/24	20	0	0	0	0
EXSK1_020624B	2/6/24	20	0	0	0	28.95
EXSK2_020624A	2/6/24	31	0	0	23.01	25.27
EXSK2_020624B	2/6/24	31	0	0	0	0
EXSK2.5_020624A	2/6/24	145	24.64	0	23.21	18.73
EXSK2.5_020624B	2/6/24	145	0	0	0	21.3
EXSK3_020624A	2/6/24	31	0	24.03	0	0
EXSK3_020624B	2/6/24	31	0	NA	24.07	0

Table 2. Biomeme Protocol Optimization: determining the appropriate dilution for measuring Total Coliforms with the Biomeme, then comparing it with the IDEXX Total Coliforms.

Sample	Sample Date	IDEXX Total Coliforms	Biomeme Human Fecal 1 µl	Biomeme Human Fecal 5 µl	Biomeme Human Fecal 10µl	Biomeme Human Fecal 20µl
EXSK1_020624A	2/6/24	2489	26.88	27.94	23.79	24.31
EXSK1_020624B	2/6/24	2489	28.62	0	25.06	23.17
EXSK2_020624A	2/6/24	1309	28.79	27.06	24.65	22.63
EXSK2_020624B	2/6/24	1309	27.74	26.22	24.65	22.85
EXSK2.5_020624A	2/6/24	24196	23.49	21.37	19.26	18.04
EXSK2.5_020624B	2/6/24	24196	23.71	21.75	21.64	0

EXSK3_020624A	2/6/24	1376	30.52	27.2	25.53	26.27
EXSK3_020624B	2/6/24	1376	26.36	NA	27.55	0

To test the optimized Biomeme protocol, I used the newly-extracted historical samples shown in table 4. I made note of every time the Biomeme detected *E. Coli* and Human Fecal bacteria, and compared it to the IDEXX detection of *E. Coli* and Total Coliforms when they were over the recreational water safety threshold. The recreational water criteria safety threshold for single samples is 235 CFU per 100 ml for *E. Coli* and 400 CFU per 100 ml for Total Coliforms ("US EPA. Ca4-Update-Bacteria.Pdf," n.d.). I had hoped to see a correlation between the two measurements. Considering the reliability of culture-based methods such as the IDEXX, the Biomeme readings correlating would assist in confirming its use in micropollutant detection. Unfortunately, as can be seen in the table below, there was little correlation between the two methods. As this table is only taking into consideration the time when IDEXX indicated FIB concentrations over the safety threshold, I also graphed the complete IDEXX results for each site, regardless of it being over the threshold or not. To achieve the DilHFq I inverted the initial Biomeme result, then divided it by the dilution amount (either 3µl or 10µl). As the known entity, IDEXX remained on the x-axis, to be compared to the Biomeme results on the y-axis. Figure 2 graphed Total Coliforms and Human Fecal Bacteria, while Figure 3 plotted the *E. Coli* results. As seen in both figures, there was no correlation between the two measurement types.

There are several reasons why a lack of correlation could have occurred. To begin with, culture-based monitoring methods are vastly different then qPCR measurements. Furthermore, the Biomeme is only capable of reading 20 µl at most, taken from the DNA extracted after filtering only 500-750 mL of a water sample. Meanwhile the IDEXX uses between 10 mL to 100

mL of a water sample. It is possible that there was simply no bacterial DNA in the sample used for a Biomeme reading. Regardless, it was disappointing that the results were not better correlated.

Table 4. Comparing when Biomeme detects *E. Coli* and Human Fecal vs IDEXX detection of *E. Coli* and Total Coliforms over the safety threshold for single samples. There were Total Coliforms detected at each of these sites, however this chart is only making note of when those measurements exceed the safety threshold. There are times when the Biomeme accurately detects the presence of FIBs aligning with the IDEXX measurements as being over the threshold. Unfortunately there are also several sample sites which measure Total Coliforms as over the safety threshold according to the IDEXX assays, yet the Biomeme does not detect any Human Fecal bacteria. Samples with a dilution of 10 µl were run first, the 3 µl ones were run after, due to the struggle with 10 µl not being consistently read.

Site	µl Dilution	Biomeme Detected *E. Coli*	IDEXX *E. Coli* concentrations over limit?	Biomeme Detected Human Fecal	IDEXX Total Coliforms concentrations over limit?
SK1_041219	3	no	no	yes	no
SK2_041219	3	no	no	yes	no
SK2.5_041219	3	no	no	yes	yes
SK3_041219	10	yes	no	yes	no
SK4_041219	10	no	no	no	no
SK5_041219	3	no	no	no	yes
SK6_041219	3	no	no	yes	yes
SK7_041219	3	n/a	no	n/a	no
SK8_041219	3	n/a	no	n/a	no
SK9_041219	10	yes	no	yes	yes

SK10_041219	3	no	no	no	yes
SK11_041219	3	no	no	no	no
SK12_041219	3	no	no	yes	no
SK14_041219	3	no	no	no	yes
SK1_051019	3	no	no	yes	yes
SK2.5_051019	3	no	no	yes	yes
SK3_051019	3	no	no	no	no
SK4_051019	3	no	no	no	no

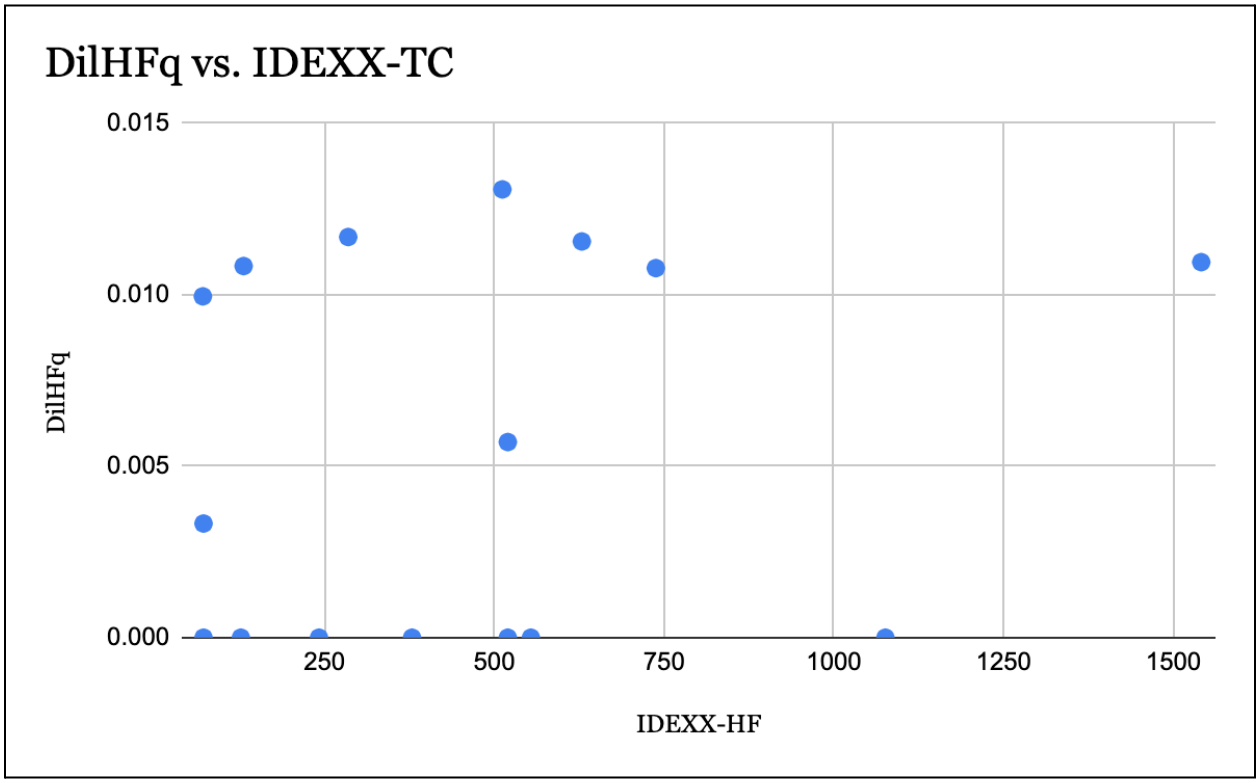

Figure 2. Plotting the relationship between the IDEXX results for Total Coliforms compared to the diluted Biomeme Human Fecal measurements. To obtain the DilHFq, the initial Biomeme result is inverted, then divided by the dilution amount. This figure is taking into account all of the IDEXX results,

regardless of if they were over the safety threshold or not. There is no clear correlation between the IDEXX and Biomeme.

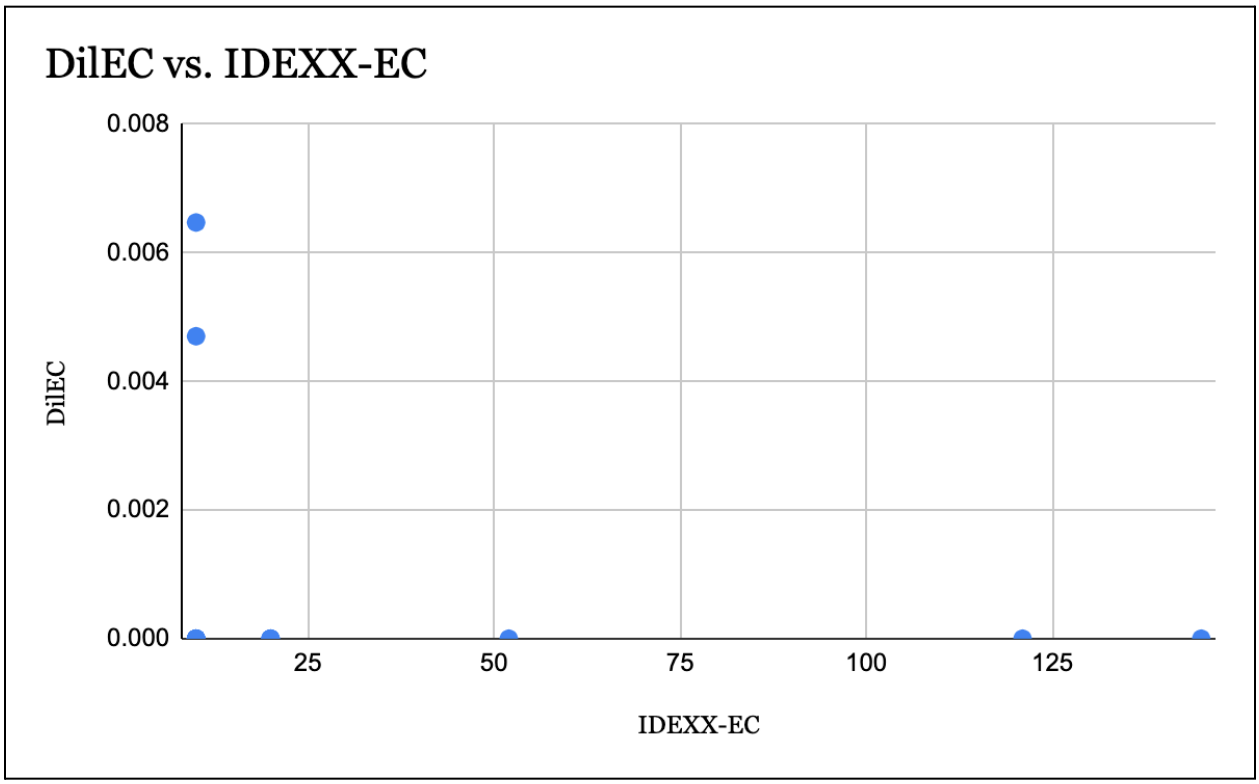

Figure 3. Plotting the relationship between the IDEXX results for *E. Coli* compared to the diluted Biomeme *E. Coli* measurements. To obtain the DilEC, the initial Biomeme result is inverted, then divided by the dilution amount. This figure is taking into account all of the IDEXX results, regardless of if they were over the safety threshold or not. There is no clear correlation between the IDEXX and Biomeme.

The dilution process proved more complicated than originally expected. In the initial Protocol Optimization it was decided that 10 µl of sample mixed with 10 µl of DNase/RNase free water worked best, as shown in tables 1 and 2. It came as a surprise when following Protocol Optimization, multiple runs would not work when using historical samples from 2019. A Biomeme test not working is signified by the Internal Positive Control (IPC). When IPC reads as 0.00, it indicates that the sample was not properly read. There are several reasons why this might be the case, one being natural inhibitors which might disrupt the process. Alongside the bacteria I was attempting to monitor, there are an abundance of various other microcontaminants that are

present in the water sample, and although the filtration process and DNA extraction should have eliminated many of these contaminants, it is possible that natural inhibitors would be interfering with the Biomeme's capabilities. The fewer of those inhibitors that are present, the more likely it is that the sample will be properly read ("BioPoo Assay on eDNA," n.d.). To get less of those inhibitors within a sample and hoping for more consistent results, I chose to change the dilution to 3 μl of sample with 17 μl of DNase/RNase free water. With this change, the IPC responded with less issues.

Unfortunately, there was little correlation between the IDEXX assay results and the qPCR readings. In part this is because culture based approaches such as the IDEXX will always be more sensitive than qPCR, as can be seen in Table 4, which shows when the IDEXX is able to detect bacteria where the Biomeme does not. It should also be considered that as a culture-based method, the IDEXX is measuring growth, while the qPCR is looking at bacteria from a genetic standpoint. These differences can make it difficult to truly consider these two different approaches as comparable (Converse et al. 2012).

It is important to note that there should be more water filtered through the Sterivex filters after sampling occurs. For the samples used, approximately 500-750 mL of water was pushed through the Sterivex filters. Is it possible that although there was significant bacteria present in the water, there was not enough filtered to accurately obtain a measurable amount for the Biomeme. The Biomeme only reads 20 μl at most, using samples that have gone through the DNA extraction process following the Sterivex filtration process. In comparison, the IDEXX uses 10 mL of water for *E.Coli* and Total Coliforms readings, and 100 mL of water sample for *Enterococcus*. Given the larger volume of water being analyzed, there may simply be more

bacteria present in the IDEXX samples. Moving forward the sampling process should require a higher volume of water during the filtration process.

Conclusion

Pathogenic bacteria are the cause for many diseases and infections around the world, however testing for each specific bacteria is a difficult and unsustainable process. Fortunately, as most of these pathogens are associated with waste, there are a few bacteria that aid in the detection and monitoring of harmful microbes. These bacteria, known as Fecal Indicator Bacteria (FIB) are currently monitored through the use of culture based methods such as the IDEXX. However, although accurate when monitoring FIB concentrations, this process is not capable of detecting antibiotic resistant genes (ARGs) in bacteria present in our waterways. Specifically, the Int1 gene should be monitored, as this an indicator for ARGs and as it allows for the transference of antibiotic resistance between bacteria. Unfortunately there are currently no reliable field ready methods for tracking the Int1 gene, although recently a new piece of technology is being considered for testing for the Int1 gene. TheBiomeme Franklin® Real-Time PCR Thermocycler is designed to detect FIBs, however it has shown potential for testing for the Int1 gene as well. After working on the optimization protocol of the Biomeme, I investigated its potential through comparison of 2019 historic samples of the Saw Kill with the IDEXX FIB measurements. Although the Biomeme was capable of detecting the presence of *E. Coli* and Human Fecal associated bacteria, the results did not correlate with the IDEXX measurements. Unfortunately, there were significant inconsistencies between the dilutions of each Biomeme run, as well as between the Biomeme results and the IDEXX results. To better determine the Biomeme's

potential for the detection of various pollutants including the Int1 gene, these inconsistencies between qPCR and culture-based methods must first be researched and resolved.

Moving forward I would suggest a study focused on what inhibitors may be interfering with the samples as they are read by the Biomeme. The decision to use 10 µl of sample and 10 µl of DNase/RNase free water was made after extensive protocol optimization work, however once historic samples were used, I ran into issues with the dilution working inconsistently. Although I was able to somewhat determine the cause as a result of inhibitors within the sample, there are still uncertainties with understanding to what extent these inhibitors interfere with the Biomeme. How might this be impacting the reliability of its readings? I would suggest further analysis of what might be present in the sample that could cause interference, compared with better understanding of how much DNA is present in the sample to begin with. Better understanding the DNA concentrations to begin with might help in knowing what to expect from the Biomeme readings. The dilution issues and inconsistencies should be addressed, and the cause should be studied further. Truly understanding what may be inhibiting the Biomeme's capability to read samples is essential for its potential application to Int1 detection.

Another study I would suggest would be comparing the Biomeme readings of a few sites over the course of several months or years. Rather than using samples from 14 different sites and comparing each of those different sites to the IDEXX, as I did in this study, it could be beneficial to focus on fewer sites. Through analyzing the results of just a few sites over a long period of time, there may be patterns that can aid in better understanding how the Biomeme works for various pollutants, including, hopefully, the Int1 gene.

The Biomeme's ease of use and quick turnaround time would be incredibly helpful in monitoring indicators varying from FIBs to the Int1 gene. It is my hope this technology can aid

studies in tracking ARGs, and while there is significant work still needed to get it to that point, I believe it to be possible.

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