QUANTIFICATION OF MULTIPLE WATERBORNE PATHOGENS IN DRINKING WATERS, DRAINAGE CHANNELS, AND SURFACE WATERS IN KAMPALA, UGANDA OVER SEVEN MONTHS OF SEASONAL VARIATION

BY

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THESIS

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ABSTRACT

Longitudinal water quality monitoring is important for understanding seasonal variations in water quality, waterborne disease transmission, and future implications for climate change and public health. In this study, microfluidic quantitative PCR (MFQPCR) was used to assess the presence of human enteric pathogens in protected springs, a public tap, drainage channels, and surface water in Kampala, Uganda from November 2014 to May 2015. Because waterborne disease incidence in Uganda has been shown to increase during the wet seasons, we assessed the differences in relative abundance of multiple waterborne pathogens during the wet and dry seasons. All water sources tested contained multiple pathogens, with drainage channels and surface waters containing higher abundance over protected springs and the public tap. Pathogens detected included Enterohemorrhagic *E. coli*, *Shigella* spp., *Salmonella* spp., *Vibrio cholerae*, and Enterovirus. Drainage channels were found to be significantly more contaminated during the wet season compared to the dry season, whereas drinking water sources contained little to no seasonal variation. These results suggest that individual water source types respond uniquely to seasonal variability, and that human interaction with contaminated water sources, rather than direct ingestion, is a major contributor to waterborne disease transmission. These findings direct public health and climate change adaptation efforts towards sanitation, solid waste management, and education about water and food safety.

To the Sadik family – Mom, Dad, Jacob, Adam, and Sarah

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CHAPTER 1: INTRODUCTION

Globally in 2010, diarrheal diseases were responsible for 810,000 deaths among children under the age of five, with about 90% of these deaths occurring in Sub-Saharan Africa and South Asia (1). It is estimated that approximately 88% of all diarrhea-attributable diseases are preventable through safe water, sanitation, and hygiene (2). While the Millennium Development Goal which aimed to have 88% of the global population with access to improved drinking water was met in 2010 (3), climate change introduces unique challenges in creating safe and resilient water sources necessary in maintaining and surpassing development goals. As the climate changes, higher average sea and ambient temperatures, as well as frequency of flood and drought events are projected to increase, putting pressure on water resources for drinking and agriculture, and posing public health risks (4-7). Developing effective climate change adaptation strategies is critical to maintaining economic stability, food security, and public health, but data to guide adaptation is limited and largely uncertain (8-10). To provide robust climate change recommendations for public health, water, and sanitation sectors, the epidemiological relationship between local climate and waterborne disease must be well understood (8, 11). Furthermore, this relationship must be well understood especially in developing countries, where climate change is argued to have the greatest impact on water and food security, and public health (4, 5). Previous research has emphasized the importance of empirical field-based measurements for designing effective climate change interventions (12, 13). We hypothesized that field-based water quality measurements would provide the greatest impact if assessed over time to capture seasonal variability in water quality. One of the goals of the study presented here is to provide empirical seasonal water quality data that may contribute to continued climate and

waterborne disease analysis, particularly for Kampala, Uganda and other developing tropical regions.

The population of Uganda is approximately 34.9 million people and is growing nationally at a rate of 3.0% (14), and in urban areas at a rate of 5.6% (14, 15). The rapid urbanization of Kampala, exacerbated by poverty and inadequate physical planning, has given rise to the expansion of informal settlements, substandard housing often subjected to overcrowding, poor water and sanitation conditions, and limited access to basic health, energy, and security services. Populations that reside in informal settlements are the most at risk to water and food insecurity, infrastructure failure, and disease transmission (15-17). A national report in 2008 concluded that 63.1% of households in slum settlements of Uganda do not have access to designated solid waste locations (18). The health hazards posed by poor solid waste management are severely compounded by the lack of improved water access, with only 13.9% of informal settlement residents in Uganda having access to piped water (18). Limited access to safe water due to lack of coverage and high costs (19) forces urban residents to collect water from contaminated water sources and as a result, urban areas are frequently afflicted with waterborne diseases such as cholera, dysentery, cryptosporidiosis, and rotavirus (20-23). Waterborne disease prevalence in Uganda has characteristically increased immediately before and during the rainy season (17, 21, 24), suggesting that seasonal variability is an important component of disease transmission in Uganda. Furthermore, the effects of seasonal variability on water quality may provide insight about the implications of climate change with respect to water quality and waterborne disease transmission. Given that climate change is likely to exacerbate health risks in urban poor populations due to inadequate economic capacity, infrastructure resilience and health services (4,

25), studying microbial reservoirs, such as community water sources, that harbor pathogenic microorganisms, is imperative.

The climate change preparation capacity for Kampala, Uganda would likely be enhanced by an increased and longitudinal understanding of water quality in urban communities. Currently, much biological monitoring of water sources in the developing world is focused on culture-based enumeration of fecal indicator bacteria (FIB) such as *E. coli*, fecal coliforms, and thermotolerant coliforms (26-29). However, numerous publications have shown that FIB do not consistently or precisely represent the presence of bacteria, viruses, and protozoa (30-32) particularly in tropical environments with higher temperatures and greater nutrient and organic matter content, and in situations of non-point source contamination (33, 34). High-sensitivity and high-throughput methods to monitor water quality over the long-term is necessary to observe seasonal variation of water quality. Furthermore, efficient and representative water quality monitoring in developing regions will have a greater global impact on improving water security in the context of climate change. Microfluidic quantitative polymerase chain reaction (MFQPCR) is a high-throughput chip-based PCR assay that is a promising monitoring tool for global biological water quality. MFQPCR utilizes microfluidics technology to increase sensitivity and specificity, and reduce cost, reagent and sample consumption, and time compared to conventional singleplex and multiplex qPCR (35). The high-throughput nature of this method allows for rapid and thorough detection of up to 96 target sequences in 96 samples, performing over 2,000 qPCR reactions in parallel. Previous environmental water studies have shown that MFQPCR maintains and may surpass overall sensitivity compared to conventional qPCR (36, 37). Applying this method to water quality analysis results in the simultaneous detection of multiple waterborne pathogens across multiple water samples (37-39).

The objectives of this study were 1) to identify and quantify human enteric pathogens in drinking water, drainage channels, and surface water over seven months during seasonal variation in Kampala, Uganda and 2) to demonstrate the use of MFQPCR as an effective long-term biological water quality monitoring tool. Water samples were collected from drinking water, drainage channels, and surface water in Kampala, Uganda from November 2014 through May 2015. The variation of waterborne pathogen presence among water sources was measured over time. To the best of our knowledge, this is the first study to longitudinally measure waterborne pathogen presence in multiple water source types in a developing tropical region.

CHAPTER 2: MATERIALS AND METHODS

2.1 Location and Sampling

The city of Kampala is comprised of five administrative divisions: Central, Kawempe, Nakawa, Makindye, and Rubaga. Water samples from eight protected springs, one treated public tap, two drainage channels, and one lake were collected from November 2014 through May 2015. These water source types were selected to monitor the variation of water quality parameters as well as pathogen presence among different water sources over time. Water sources in informal settlements and densely populated neighborhoods were selected over water sources in neighborhoods with lower population density. Six neighborhoods in the Central, Kawempe, Makindye and Nakawa divisions were chosen due to proximity between sampling sites and capacity to process samples. Sampling locations and water source types are indicated in Figure 1.

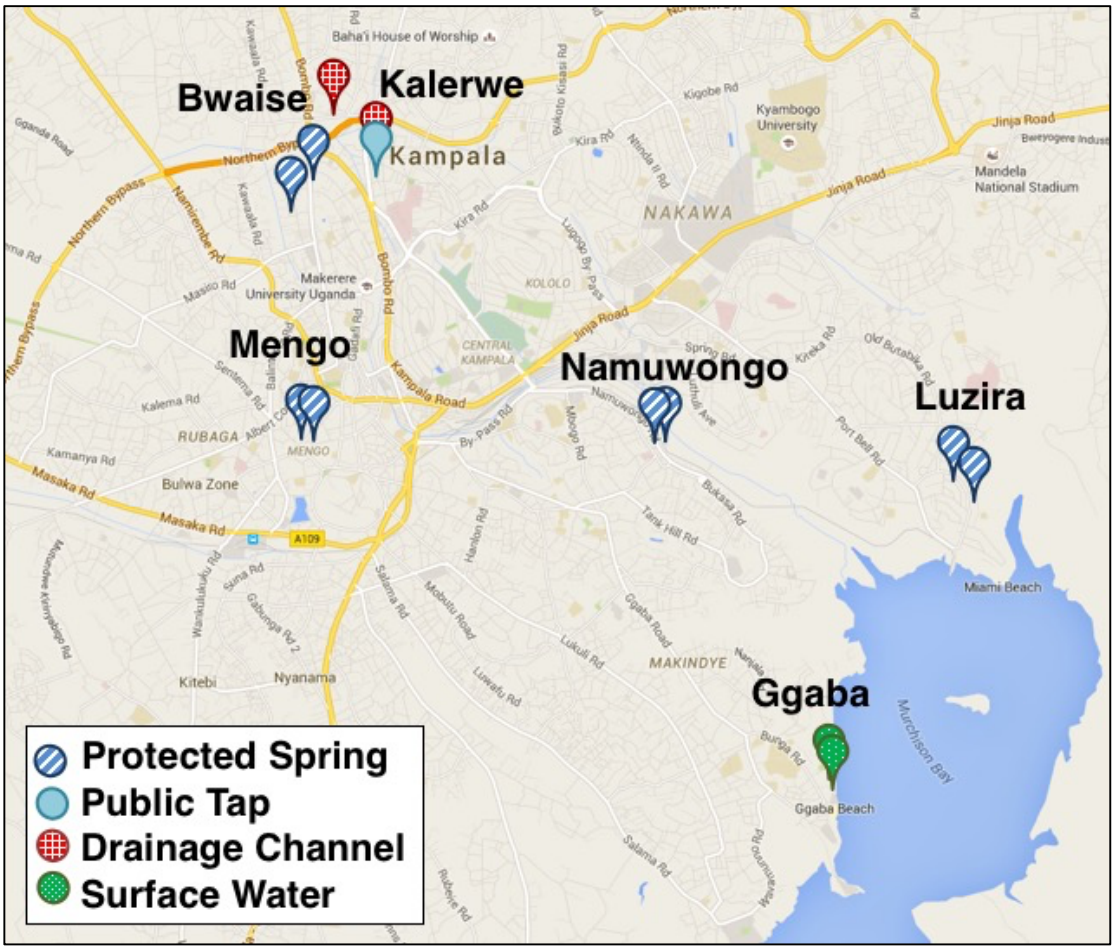

Figure 1. Sampling map indicates the location of all sampling sites and water source types. a) Bwaise contained two protected springs (B1, B2) and one drainage channel (B3). b) Kalerwe contained one drainage channel (Ka1) and one public tap that is treated drinking water supplied by the National Water and Sewerage Corporation (Ka2). c) Luzira contained two protected springs (L1, L2). d) Mengo contained 2 protected springs (M1, M2). e) Namowongo contained two protected springs (N1, N2). f) Ggaba contained a lake with two sampling locations (G1, G2).

Two-liter water samples were collected from protected springs and the public tap using reusable sterilized bags (Boli, Zhejiang, China). Bags were sterilized 24h prior to sample collection according to EPA guidelines (40). Drainage channel and Lake Victoria water samples were collected in 0.5 L aliquots in sterile Whirlpak (Nasco, Fort Atkinson, WI) bags. Upon collection, all samples were stored at 4°C and were processed within 24h. The dates and seasons of collected samples are summarized in Table 1.

Table 1. Summary of collected water samples. Season was determined based on supporting literature (41, 42).

Sample Set	Dates Collected	Season
SS1	10 Nov - 20 Nov (2014)	Wet
SS2	24 Nov - 5 Dec (2014)	Wet
SS3	4 Dec - 18 Dec (2014)	Wet
SS4	6 Jan - 7 Jan (2015)	Dry
SS5	19 Jan - 23 Jan (2015)	Dry
SS6	3 Feb -10 Feb (2015)	Dry
SS7	17 Feb - 2 Mar (2015)	Dry
SS8	3 Mar- 17 Apr (2015)	Wet
SS9	22 Apr - 11 May (2015)	Wet
SS10	20 May - 27 May (2015)	Wet

Water samples were concentrated onto 47 mm diameter filters to capture and preserve microorganisms for downstream qPCR and MFQPCR. Within 24h of collection, water samples were treated with sterile 25mM $MgCl_2$-$6H_20$ (Sigma-Aldrich, St. Louis, MO) for 30 min with periodic mixing to coagulate particles and microorganisms (43, 44). Thereafter, water samples were sequentially vacuum filtered through a 1.6 μm glass fiber filter (Millipore, Ballerica, MA) followed by a 0.45 μm nitrocellulose filter (GVS Maine, Sanford, ME) placed in a 47-mm filtration funnel (Pall Corporation, New York, NY) (45). The filtration housing and flasks to catch filtrate were sterilized prior to each sample filtration according to EPA guidelines (40). Filters were treated with 500 μl RNAlater (Qiagen, Helden, Germany) to preserve microbial genomes and were stored in sterile Whirpak (Nasco) bags at -20°C until transport to University of Illinois (UIUC). At UIUC, filters were stored at -80°C until DNA/RNA extraction.

2.2 Genome Extraction and cDNA Synthesis

1.6 μm membrane filters from protected springs in the same community (B1, B2; L1, L2; N1, N2; M1, M2) and Lake Victoria water samples (G1, G2) were combined during extraction to expedite sample processing and pathogen enumeration. 0.45 μm filters were similarly combined for protected springs and Lake Victoria water samples. 1.6 μm and 0.45 μm membrane filters from drainage channel water samples and public tap water samples (B3; Ka1; Ka2) were extracted individually. Table 2 shows the manner in which samples were combined and the distances between the water sources of combined samples.

Table 2. Membrane filters from protected spring and Lake Victoria water samples were combined during genome extraction. Membrane filters from drainage channels and the public tap were genome extracted individually.

Water Source Type	**Sample Name (Pre)**	**Sample Name (Post)**	**Distance Between Two Water Sources (km)**
Protected Spring	B1	B12	0.21
	B2		
Surface Water	B3		Single Site
Surface Water	Ka1		Single Site
Public Tap	Ka2		Single Site
Protected Spring	L1	L12	0.014
	L2		
Lake Victoria	G1	G12	0.068
	G2		
Protected Spring	N1	N12	0.14
	N2		
Protected Spring	M1	M12	0.052
	M2		

Two extraction methodologies were applied due to the difference in the two membrane filter materials. The 1.6 μm glass fiber filters were extracted for DNA only using the FastDNA Spin Kit for Soil (MP Biomedicals, Santa Ana, CA) according the manufacturer's instructions

with minor modifications. The 0.45 μm nitrocellulose filters were extracted for RNA and DNA with the PowerWater RNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA) according to the manufacturer's instructions with minor modifications. All DNA/RNA genome extracts were stored at -80°C.

DNA/RNA extracts eluted from the 0.45 μm nitrocellulose filters underwent reverse transcription (RT) prior to enumeration by MFQPCR. cDNA synthesis was performed in a MyCycler Thermal Cycler (Bio-Rad, Hercules, CA) using the iScript cDNA Synthesis Kit (Bio-Rad). The reaction mixture (20 μl) contained 5X iScript Reaction Mix, 1 μl iScript Reverse Transcriptase, and 2 μl template DNA/RNA, and was performed under the following thermal cycle conditions: 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min. cDNA/DNA samples were stored at -20°C.

2.3 Conventional qPCR and MFQPCR Assays

The objective of the MFQPCR assay was to detect 21 genes corresponding to 14 total pathogens including 6 viruses, 7 bacteria, and 1 protozoon, and one nonpathogenic bacteria used as an internal control. Assay designs for all target genes were adapted from previous studies (38, 39, 46-55) to detect Adenovirus types 40 and 41, Enterovirus, Human Norovirus types GI and GII, Rotavirus Group A, Hepatitis A Virus (all genotypes), Hepatitis E Virus (all genotypes), General *E. coli*, Enterohaemorrhagic *E. coli* (EHEC), *Shigella* spp., *Salmonella* spp., *Campylobacter jejuni, Legionella pneumophila, Vibrio cholerae, Cryptosporidium,* and *Pseudogulbenkiania* sp. NH8B. A list of target genes and primer and probe sequences are summarized in Table 3.

Forward and reverse primers for all assays were obtained as Custom DNA Oligos (Integrated DNA Technologies, Coralville, IA). Three fluorogenic probe types were used as indicated in Table 3. Double quenched hydrolysis probes were labeled with 6-fluorescein (6-FAM) at the 5' end, Iowa Black FQ quencher at the 3' end, and an internal ZEN quencher located 9 nucleotides from the 5' end (Integrated DNA Technologies). TaqMan hydrolysis probes were labeled with 6-FAM at the 5' end, and nonfluorescent quencher and minor groove binder (NFQ-MGB) at the 3' end (Thermo Fisher Scientific, Waltham, MA). Probes obtained from the Universal Probe Library (UPL) (Roche, Basel, Switzerland) were labeled with 6-FAM at the 5' end and a dark quencher dye at the 3' end and contained a short sequence (8-9 nucleotides) of locked nucleic acids (56).

Plasmid standards were graciously obtained from Dr. Satoshi Ishii (University of Minnesota, St. Paul, MN) and Dr. Daisuke Sano (Hokkaido University, Sapporo, Japan) and were transformed into *E. coli* JM109 (Promega, Madison, WI) chemically competent cells or *E. coli* BL21 (DE3) (Promega) electrocompetent cells using an electroporator (Bio-Rad). Plasmids were linearized using the Plasmid Miniprep Kit (Qiagen) and were quantified using Qubit fluorometric quantitation (Thermo Fisher) prior to use in qPCR and MFQPCR. All other standards were obtained as gBlock Gene Fragments (Integrated DNA Technologies). Standard curves were generated by qPCR using serial dilutions (3×10^0 to 3×10^6 copies/µl) of a standard pool containing 17 plasmid DNA and 4 gBlock DNA standards to validate the assays prior to use in MFQPCR.

The average efficiency achieved by conventional qPCR for standard curves of plasmid standards and gBlock standards was 103% ± 12.3% (n=17) and 96.8% ± 10.1% (n=4), respectively. The average lower limit of detection for plasmid standards and gBlock standards

was 46 ± 8.1 copies/μl and 47± 12 copies/μl, respectively. Conventional TaqMan real-time qPCR was performed using a MiniOpticon Real-Time PCR System (Bio-Rad). The final reaction mixture (20 μl) contained 2X TaqMan Universal PCR Master Mix (Thermo Fisher), 500 nM of each forward/reverse primer, 250 nM hydrolysis probe, and 3 μl template DNA/cDNA. qPCR reactions were conducted under the following thermal conditions: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, and 60°C for 1 min.

Prior to enumeration by MFQPCR, all cDNA/DNA samples and standard pool dilutions underwent a standard target amplification (STA) PCR to increase template DNA yields. Standard pool dilutions (3 x 10^0 to 3 x 10^6 copies/μl) amplified in the 14-cycle STA were used to generate standard curves for MFQPCR. 20X assays (18 μM of each primer and 5 μM probe) were pooled using 1 μl per assay and 179 μl of DNA Suspension Buffer (Teknova, Hollister, CA) to make a 0.2X TaqMan primer probe mix. The reaction (5 μl) contained 2.5 μl 2X TaqMan PreAmp Master Mix (Thermo Fisher), 0.5 μl 0.2X TaqMan primer probe mix and 1.25 μl of template cDNA/DNA. The PCR plate was processed with the following thermal cycle on an MJ Research Tetrad thermal cycler (MJ Research, Waltham, MA): 95°C for 10 min, and 14 cycles of 95°C for 15 sec and 60°C for 4 min. The STA products were diluted 5-fold with 20 μl of nuclease free water and were used for MFQPCR.

The sample premix (5 μl) contained 2.5 μl 2X TaqMan Master Mix, 0.25 μl 20X Gene Expression Sample Loading Reagent (Fluidigm, South San Francisco, CA), and 2.25 μl 5-fold diluted STA product. The assay mix (5 μl) contained 2.5 μl 2X Assay Loading Reagent (Fluidigm) and 2.25 μl 20X TaqMan primer probe mix. Aliquots (5 μl) of each sample and quadruplicates of each assay were loaded onto a 96.96 chip (Fluidigm). MFQPCR was

performed in a Biomark HD Real-Time PCR (Fluidigm) using the following thermal conditions: 70°C for 30 min, 25°C for 10 min, 95°C for 1 min, followed by 35 cycles of 96°C for 5 sec and 60°C for 20 sec. ROX was used as a passive dye reference.

2.4 Genome Extraction, RT and qPCR Inhibition Analysis

Control experiments were conducted to measure the efficiency of genome extraction, and to determine whether potential inhibitors present in water sample extracts had an effect on cDNA synthesis (reverse transcription) and qPCR.

Reverse transcription (RT) inhibition was evaluated by comparing RT and qPCR in extracted environmental samples, which contained both DNA and RNA, and in nuclease free water. Human rotavirus Wa was obtained and propagated as described elsewhere (57). Virus particles were extracted using the E.Z.N.A. Total RNA Kit I (Omega Bio-tek, Norcross, GA) and quantified using Qubit fluorometric quantitation (Thermo Fisher). Human rotavirus Wa extracts (<0.2 ng/μl) were spiked into nuclease free water and Kampala water sample extracts. cDNA synthesis and conventional qPCR were run in parallel as described previously. Following enumeration, quantification cycle (C_q) values from nuclease free water versus environmental water were compared for statistical differences using a two-way ANOVA in Origin 2016. Differences in standard curves was determined to be significant for p-values less than 0.05.

PCR inhibition was evaluated for the STA and MFQPCR analysis by including an internal amplification control (IAC) in environmental sample extracts. Serial dilutions of *Pseudogulbenkiania* NH8B standard were spiked into 1.6 μm sample extracts and 0.45 μm sample extracts prior to STA and MFQPCR. Following enumeration, IAC and *Pseudogulbenkiania* NH8B standard C_q values were compared for statistical differences using a

two-way ANOVA in Origin 2016. Differences in standard curves was determined to be significant for p-values less than 0.05.

Genome extraction was evaluated by measuring an internal control before and after the extraction procedure. *Pseudogulbenkiania* sp. NH8B bacteria was graciously obtained from Dr. Satoshi Ishii (University of Minnesota, St. Paul, MN) and was grown in R2A agar (Sigma-Aldrich) with a kanamycin antibiotic marker (Sigma-Aldrich). *Pseudogulbenkiania* was spiked directly onto membrane filters prior to genome extraction to achieve a final concentration of 2 x 10^5 particles/µl in 100 µl of eluate assuming 100% extraction recovery. Extracted samples and known concentrations of *Pseudogulbenkiania* sp. NH8B standard were enumerated using conventional qPCR. Extraction efficiency was calculated by dividing the fraction of quantified *Pseudogulbenkiania* by the theoretical concentration assuming 100% efficiency.

2.5 qPCR and MFQPCR Data Analysis

Quantification cycle (C_q) values and standard pool dilutions (log copies/µl) were used to generate standard curves for each assay. C_q values were determined by Bio-Rad CFX Manager software (Bio-Rad) and Real-Time PCR Analysis software (Fluidigm) for qPCR and MFQPCR, respectively. Linear regression analysis was performed to fit standard curves and calculate the goodness of fit (r^2). Assay efficiencies were calculated based on the slopes of the standard curves for each qPCR and MFQPCR assay to validate adequate target amplification (58). Standard curves were accepted as quantifiable if the efficiency achieved was greater than or equal to 90% and if the lower limit of detection was less than or equal to 30 copies/µl. Data points were accepted if at least two of the four replicates were found to be positive, and if C_q values fell into the accepted standard curve range. Data points with C_q values outside of the standard curve range

(i.e. detectable but not quantifiable) were considered negative (36, 59). Positive data points detected by MFQPCR were evaluated for seasonal significance using a two-sample t-test in Origin 2016 software. Data points from each water source that contained positive detection of target genes in both seasons were compared by grouping wet season samples versus dry season samples. Positive data points that were present in only one season were not evaluated for statistical significance. Seasonal variation was determined to be significant for p-values less than 0.05.

CHAPTER 3: RESULTS AND DISCUSSION

3.1 Sensitivities of Genome Extraction and qPCR Assays

Efficiency of genome extraction methods and sensitivity of the applied RT and qPCR assays contribute to the effectiveness of detection of target sequences in environmental samples. Genome extraction efficiencies were found to be higher for the 1.6 μm filters compared to the 0.45 μm filters. The extraction efficiency achieved was 36.6% ± 14.9% (n=6) for 0.45 μm nitrocellulose filters and 71.4% ± 11.7% (n=6) for 1.6 μm glass fiber filters. These results suggest that the extraction techniques used for the 1.6 μm glass fiber filters resulted in greater retention of genomic material, or that 0.45 μm sample extracts contained more PCR inhibitors.

Reverse transcription and qPCR inhibition was evaluated to measure the presence or absence of contaminants that may interfere with RT and qPCR performance. A two-way ANOVA analysis showed that reverse transcription of RNA in environmental samples versus nuclease free water was not significantly different ($p=0.22$), suggesting that sample extracts did not contain reverse transcription inhibitors. A negative control was also included to validate that the internal control (Human Rotavirus Wa) was not previously present in the sample. The PCR inhibition analysis revealed that some environmental samples contained PCR inhibition. A two-way ANOVA analysis comparing standards versus environmental samples were not statistically different for the 1.6 μm MFQCPR plate ($p=0.203$), but were statistically different for the 0.45 μm MFQPCR plate ($p=0.01$) These findings suggest that PCR inhibitors were present in 0.45 μm sample extracts. The difference in PCR inhibitor presence between the 1.6 μm and 0.45 μm sample extracts is potentially due to the differences in genome extraction, which may result in excess PCR inhibitors, such as salts and phenol in sample extracts (60). This is consistent with

the MFQPCR results, which showed a greater number of positive data points in 1.6 μm sample extracts.

The average lower limit of quantification (LLQ) of detection for the final selected MFQPCR assays was 5 ± 8 copies/μl. This detection limit corresponds to an average LLQ of approximately 2.65-2.95 log copies/L in drinking water and 3.26-3.56 log copies/L in drainage channel waters and Lake Victoria water. These detection limits are comparable to similar studies (37-39).

3.2 Occurrence of Waterborne Pathogens

Standard curves that were accepted based on efficiency and linear dynamic range included assays detecting EHEC (*eaeA*, *stx1*, *stx2*), *Shigella* spp. (*ipaH 7.8*, *ipaH all*), *Salmonella* spp. (*invA, ttrC*), *E. coli* (*ftsZ*), *Vibrio cholerae* (*ctxA*) and Enterovirus (Table 4). The average efficiency achieved for standard curves of the final selected assays was 99.2% ± 4.96% (n=12). Assays for the detection of Rotavirus and *Legionella pneumophila* were rejected due to positive detection in negative controls indicating contamination. The standard curves and corresponding sample quantifications for Enterovirus, *Vibrio cholerae, E. coli,* and *Salmonella* spp. (*invA)* were based on data obtained from the MFQCPR chip run with 0.45 μm sample extracts. The standard curves and corresponding sample quantifications for EHEC (*eaeA,* stx2) and *Shigella* spp. (*ipaH 7.8, ipaH all)* were based on data obtained from the MFQPCR chip run with 1.6 μm sample extracts. The standard curves and corresponding sample quantification for *Salmonella spp.* (*ttrC*) and EHEC (*stx1*) were based on data obtained from each MFQPCR chip containing 1.6 or 0.45 μm sample extracts. Data was analyzed by comparing relative amounts of

target pathogens in water sources in the dry season versus the wet season. Positive data points were expressed as gene copies/L of water.

Of the accepted positive-detection data points (n=441), 14% were positive for EHEC *eaeA*, 4% were positive for EHEC stx1, 29% were positive for EHEC *stx2,* 7% were positive for *Shigella* spp. *ipaH 7.8*, 10% were positive for *Shigella* spp. *ipaH all*, 0.5% were positive for *Salmonella* spp. *invA*, 2% were positive for *Salmonella* spp. *ttrC,* 0.2% were positive for *Vibrio cholerae ctxA,* and 7% were positive for Enterovirus (Figures 2-9). The positive detection of target pathogens was found to depend on water source type. The number of positive data points was normalized for the number of water sources of the corresponding type. Of total data points in both MFQPCR chips (n=441), 12% ± 3% were positive for protected springs, 6% ± 4% were positive for the public tap, 53% ± 8% were positive for drainage channel waters, and 29% ± 1% were positive for Lake Victoria. Drainage channel waters, B3 and Ka1, contained the most target pathogens over the seven-month period, followed by Lake Victoria water samples, G12. Protected springs, B12, L12, N12 and M12, and the public tap, Ka2, contained the least number of positive data points. Performing seasonal variation analysis for drinking water samples was limited because 36% and 38% of positive data points for protected springs and public tap, respectively, were only found in one season.

Positive detection of target genes was evaluated for each season and water source (Figure 10). Positive detection of EHEC *stx2* occurred in all water sources in wet and dry seasons, indicating the widespread prevalence *stx2* containing EHEC strains in diverse water sources. Positive detection of Salmonella (*invA, ttrC*) occurred only during the wet season in afflicted water sources, and positive detection of Vibrio cholerae (*ctxA*) occurred only once during the study period.

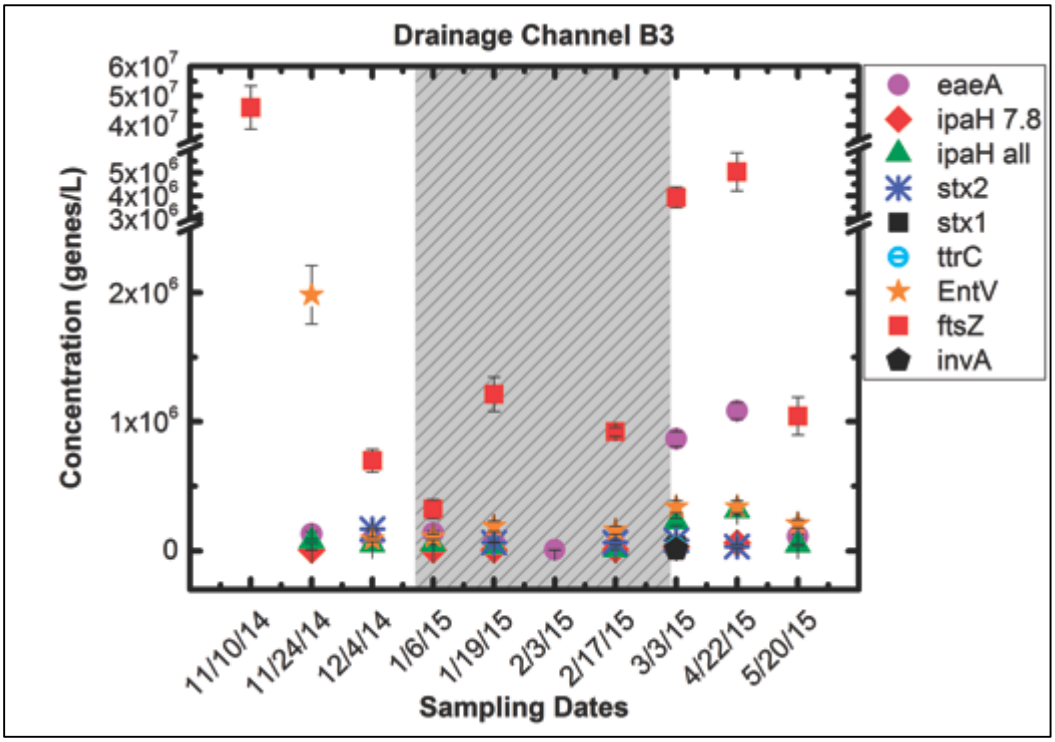

Figure 2. Waterborne pathogen presence in drainage channel B3. The shaded area indicates the dry season.

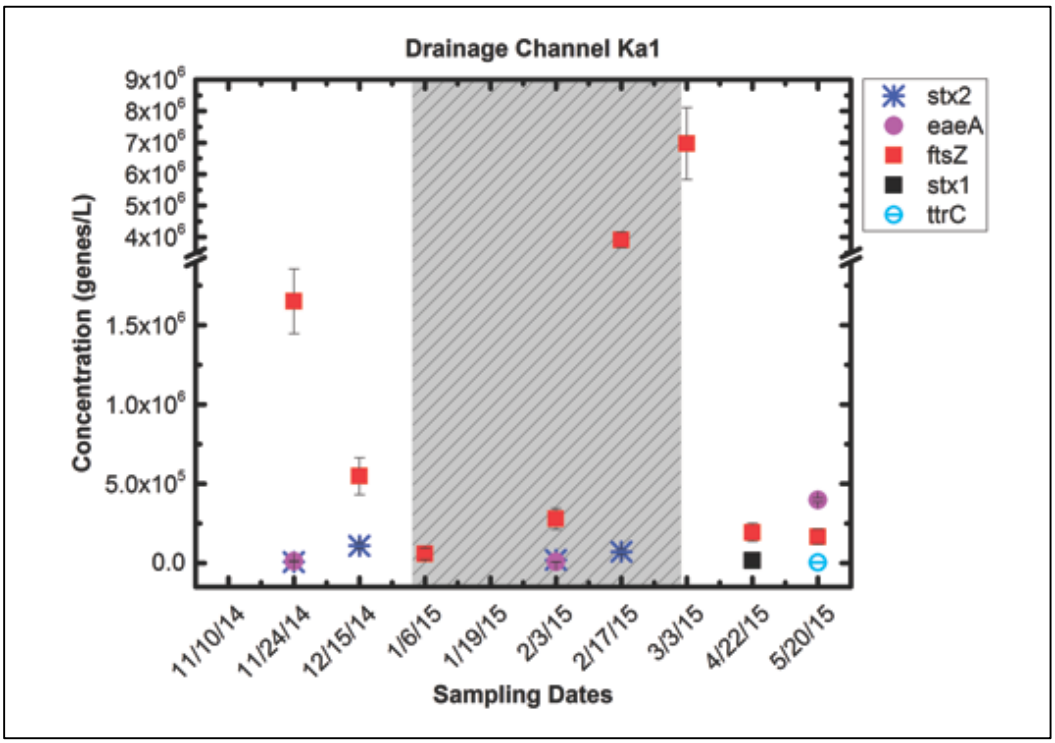

Figure 3. Waterborne pathogen presence in drainage channel Ka1. The shaded area indicates the dry season.

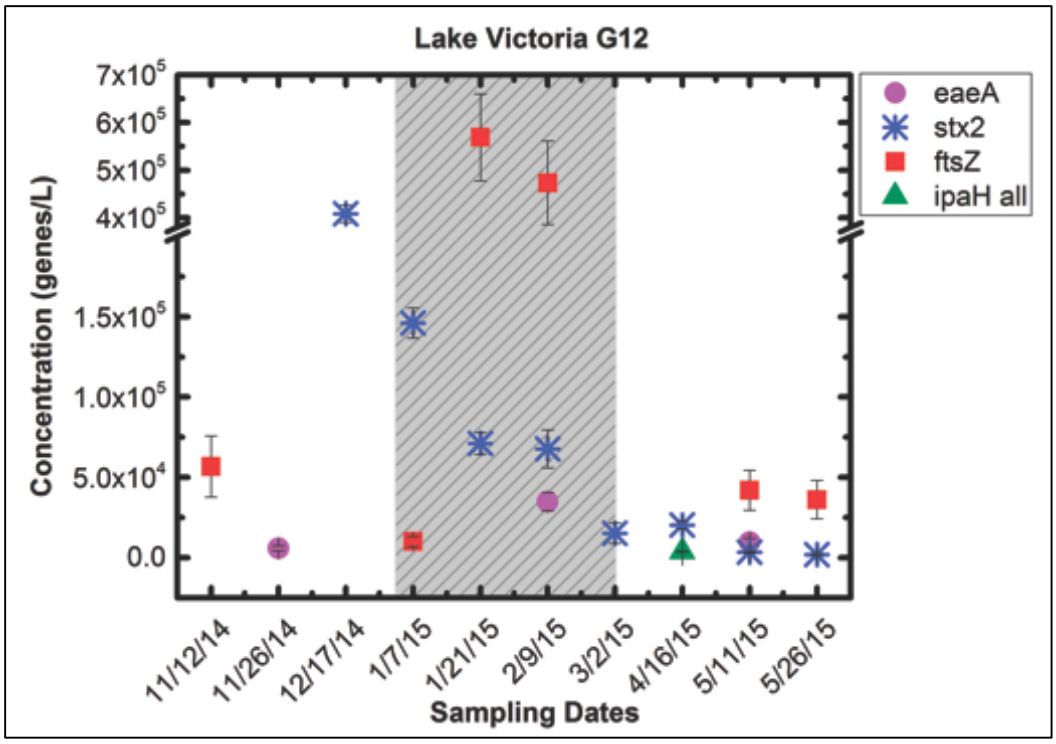

Figure 4. Waterborne pathogen presence in Lake Victoria G12. The shaded area indicates the dry season.

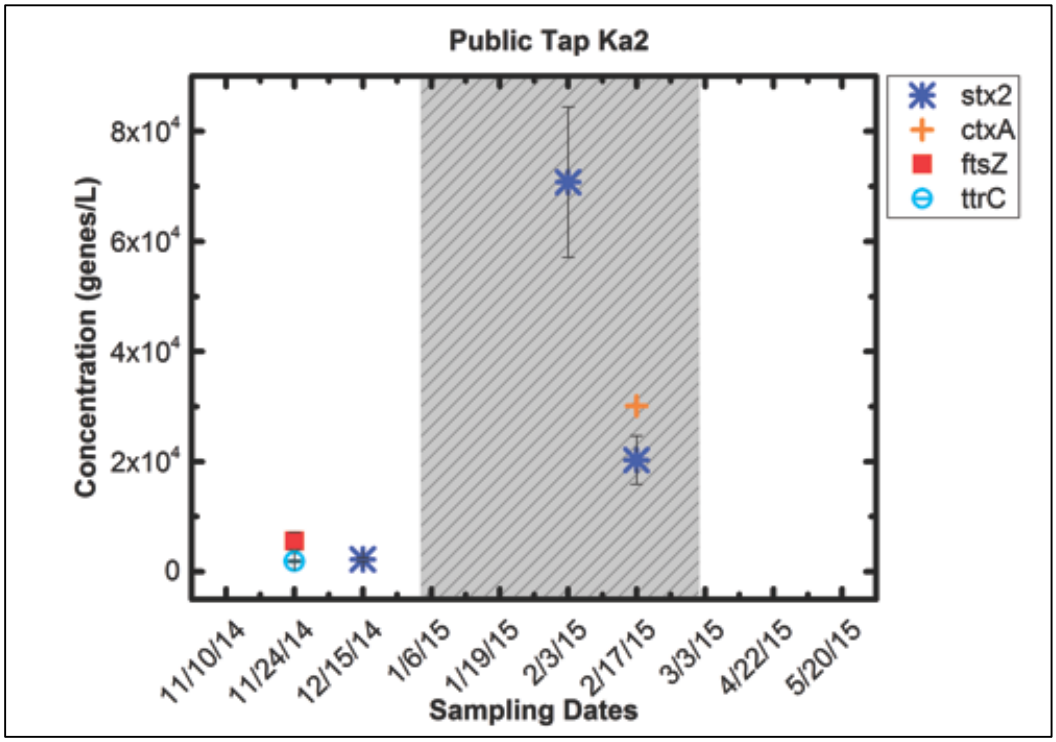

Figure 5. Waterborne pathogen presence in public tap Ka2. The shaded area indicates the dry season.

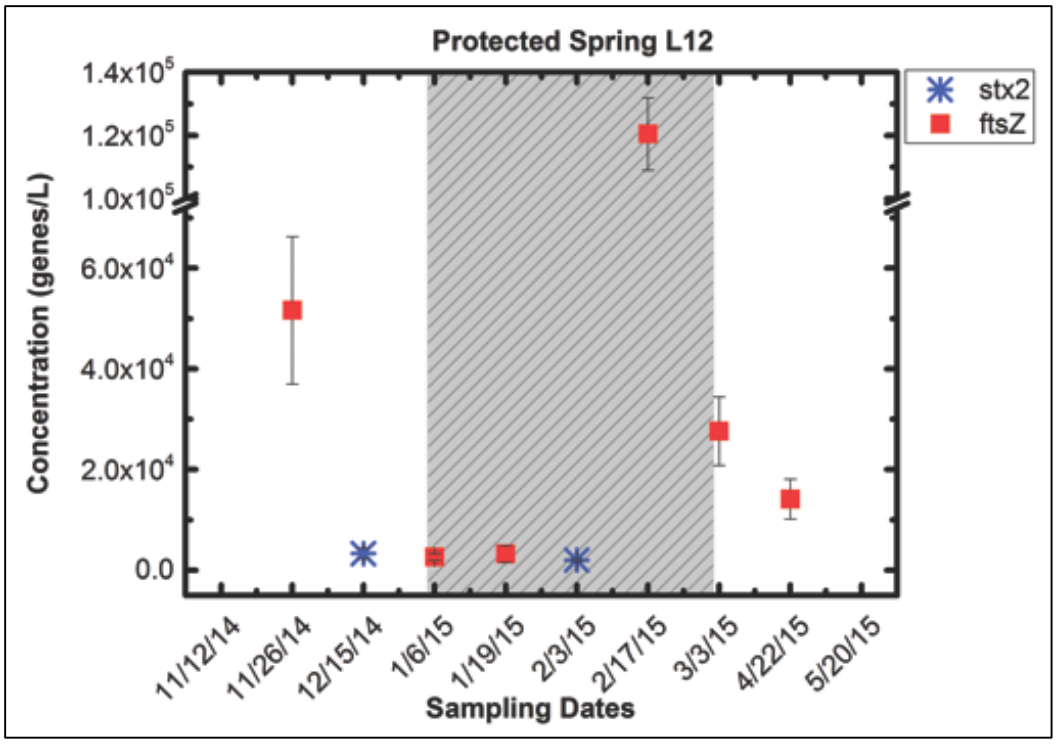

Figure 6. Waterborne pathogen presence in protected springs L12. The shaded area indicates the dry season.

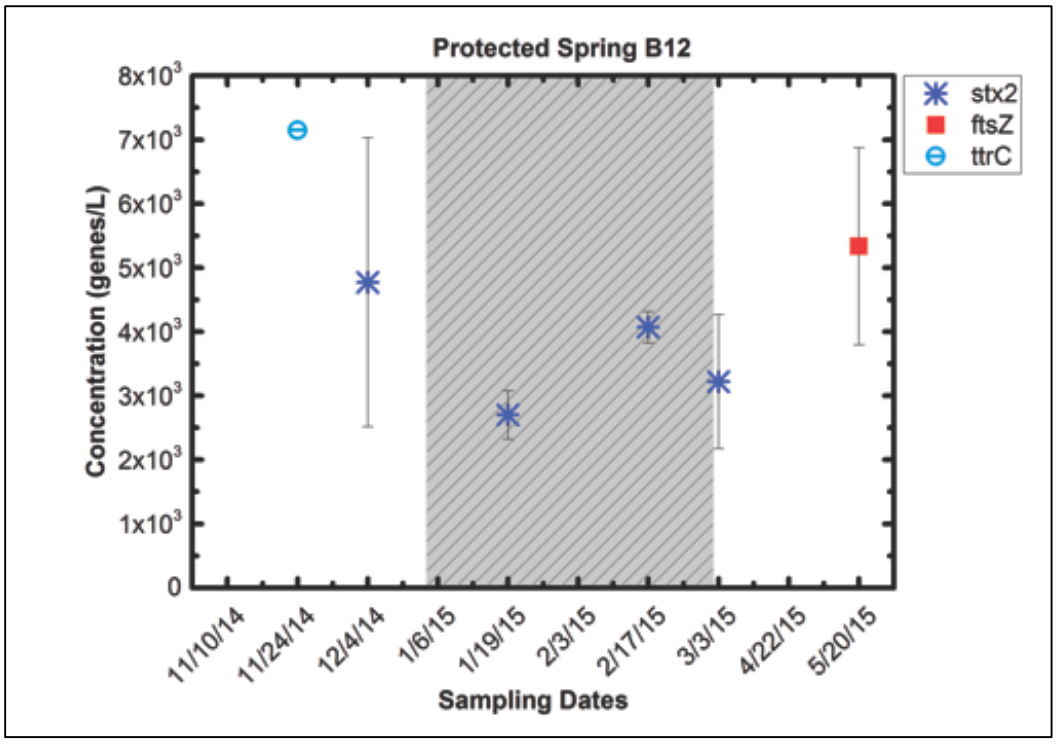

Figure 7. Target pathogen presence in protected springs B12. The shaded area indicates the dry season.

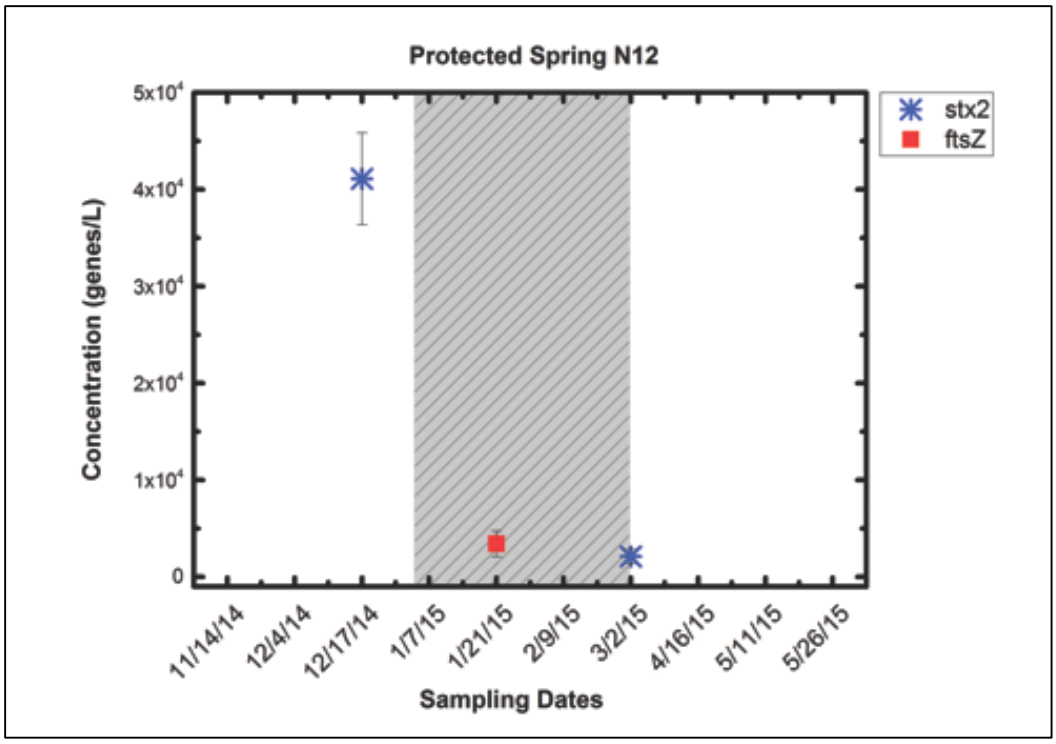

Figure 8. Waterborne pathogen presence in protected springs N12. The shaded area indicates the dry season.

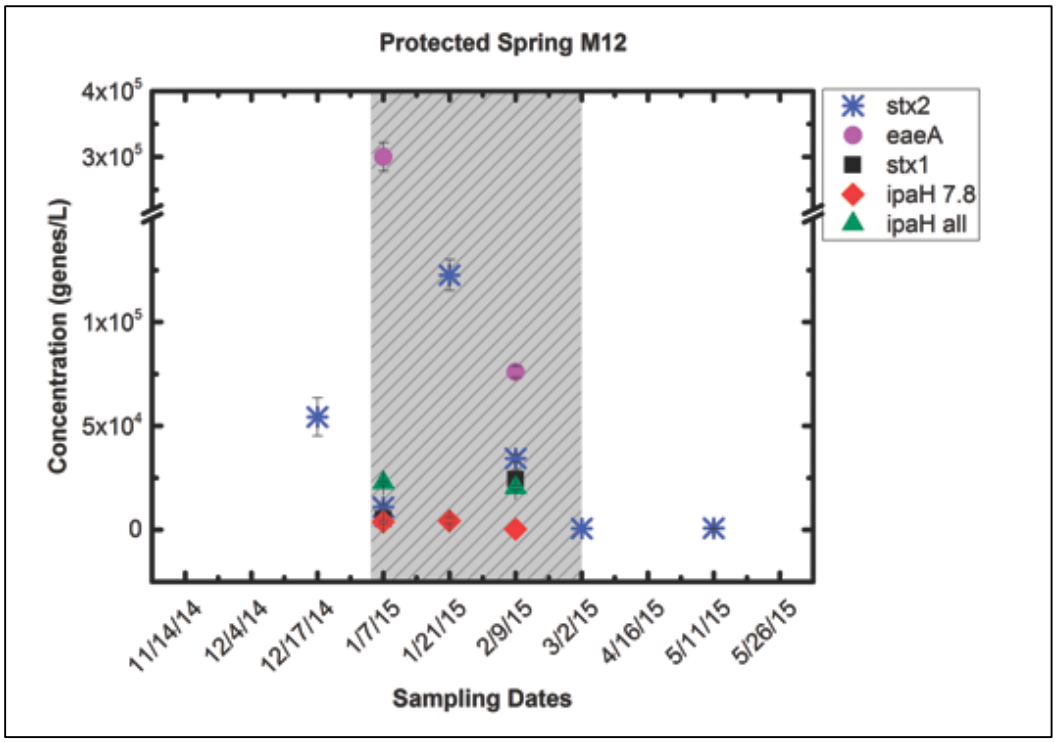

Figure 9. Waterborne pathogen presence in protected springs M12. The shaded area indicates the dry season.

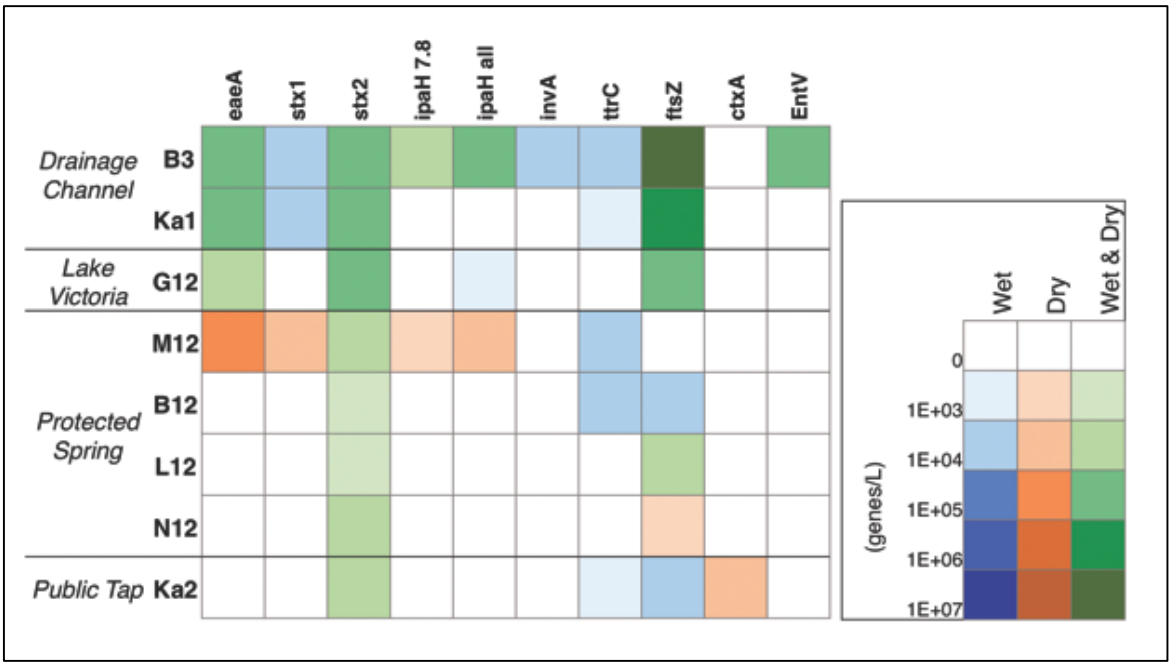

Figure 10. Abundance of target genes in water sources during the wet and dry seasons. EHEC *stx2* and *E. coli ftsZ* genes were the most prevalent across all water source types.

3.3 Seasonal Variation in Waterborne Pathogen Occurrence

Data from the MFQPCR was used to determine if seasonal variations including precipitation and runoff, among others, were attributable to observed increases or decreases in target pathogen concentrations in each water source. Overall, both drainage channels were found to contain higher concentrations of target pathogens in the wet season versus the dry season. Positive detection of *Shigella spp. ipaH 7.8* and *ipaH all,* EHEC *eaeA, E. coli ftsZ,* and Enterovirus was significantly higher in the wet season compared to the dry season for drainage channel B3 (Figure 11). Single occurrences for positive detection of EHEC *stx1* and *Salmonella* spp. *invA* and *ttrC* in B3 during the wet season only (Figures 2, 10) support this data analysis. Drainage channel Ka1 showed similar trends for the presence of EHEC *eaeA* (Figure 12), but did not exhibit statistically significant seasonal trends for the presence of *E. coli ftsZ* (p=0.55) and EHEC *stx2* (p=0.26). While both *eaeA* and *stx2* genes are used for the detection of EHEC, both genes are not present in all strains of EHEC, potentially resulting in the differences observed

(61). Similar to drainage channel B3, single occurrences for positive detection of EHEC *stx1* and *Salmonella* spp. *ttrC* was observed in the wet season only (Figures 3, 10).

Positive detection of EHEC *eaeA* and *E. coli ftsZ* in Lake Victoria water samples was significantly higher in the dry season compared to the wet season (Figure 13). However, positive detection of EHEC *stx2* gene in Lake Victoria water samples was not significantly higher in either season (n=29, p=0.31), and Shigella spp. *ipaH all* was observed as a single occurrence during the wet season (Figures 4, 10).

The public tap was observed to have significantly higher positive detection of EHEC *stx2* gene during the dry season compared to the wet season (n=12, p=0.004) as shown in Figure 14. *Salmonella* spp. *ttrC* and *E. coli ftsZ* were observed only in the wet season, and *Vibrio cholerae ctxA* was observed only in the dry season. Observations of *ttrC*, *ftsZ* and *ctxA* were all single occurrences (Figures 5, 10).

Protected springs containing positive detection of pathogenic microorganisms in wet and dry seasons were not found to have statistically significant seasonal variation (Figure 15). Statistical analysis comparing seasonal flux could not be performed for data points showing positive detection in only one season. For positively detected protected spring water samples (n=116), 5% of data points contained positive detection in the wet season only, 31% of data points contained positive detection in the dry season only, and 64% of data points contained positive detection in the wet and dry seasons.

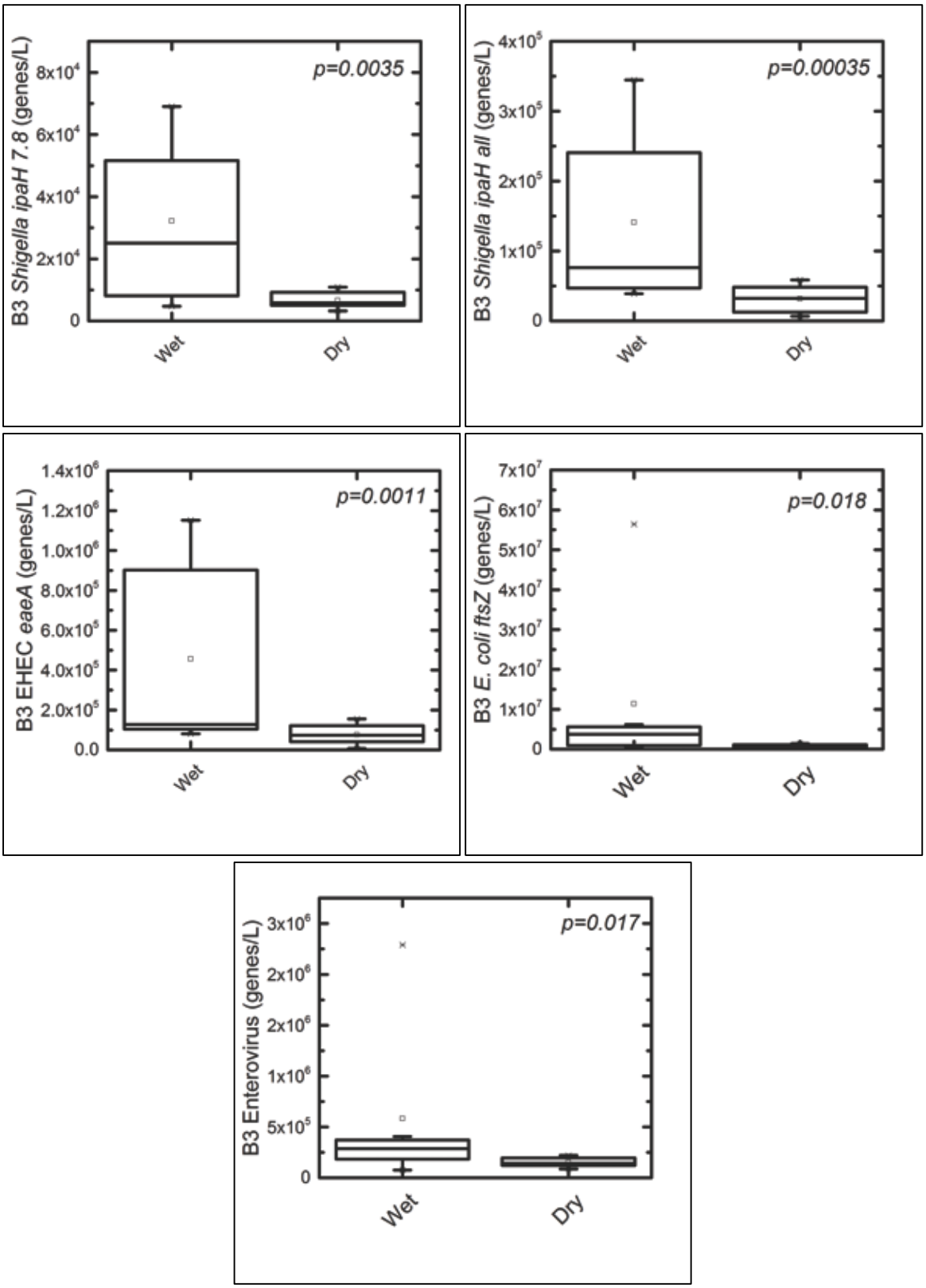

Figure 11. The concentrations of *Shigella* spp., EHEC, *E. coli,* and Enterovirus in drainage channel B3 suggest that pathogen loading increases during the wet season compared to the dry season.

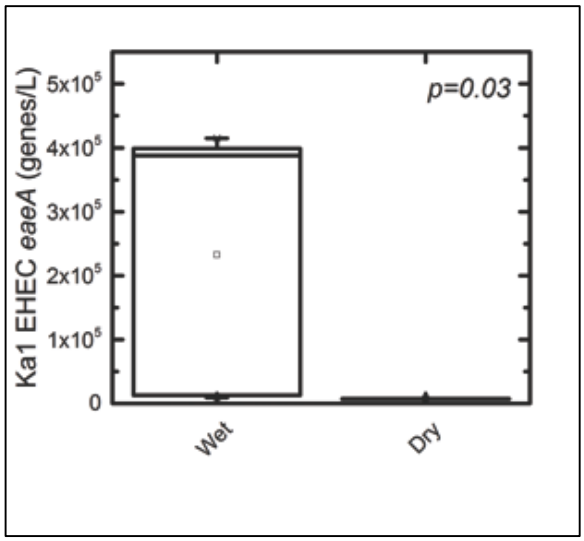

Figure 12. The concentrations of EHEC *eaeA* in drainage channel Ka1 suggest that pathogen loading increases during the wet season compared to the dry season.

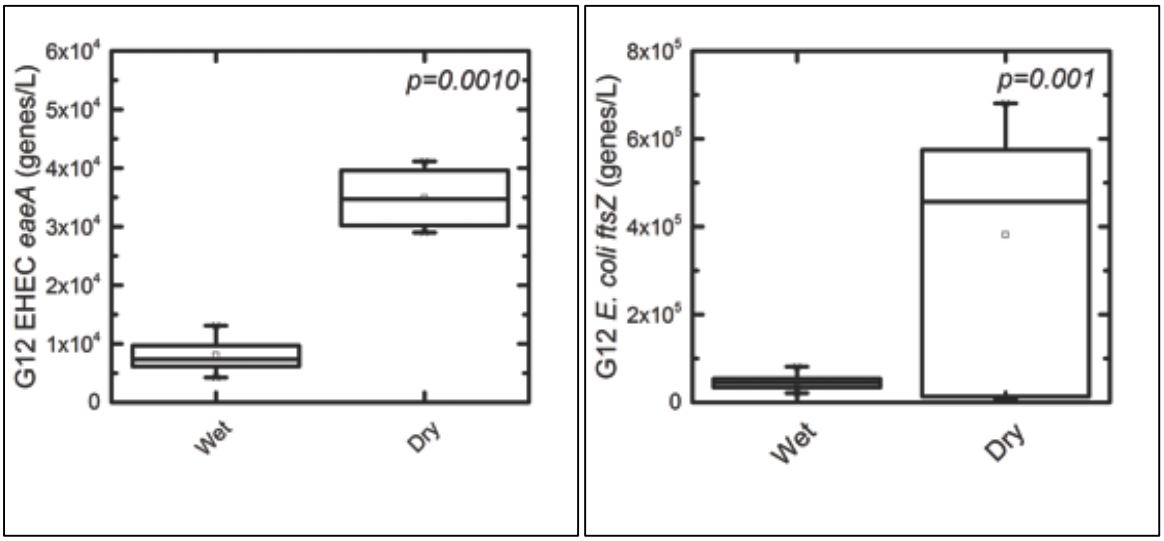

Figure 13. The concentrations of EHEC *eaeA* and *E. coli ftsZ* in Lake Victoria suggest that pathogen loading increases during the dry season compared to the wet season.

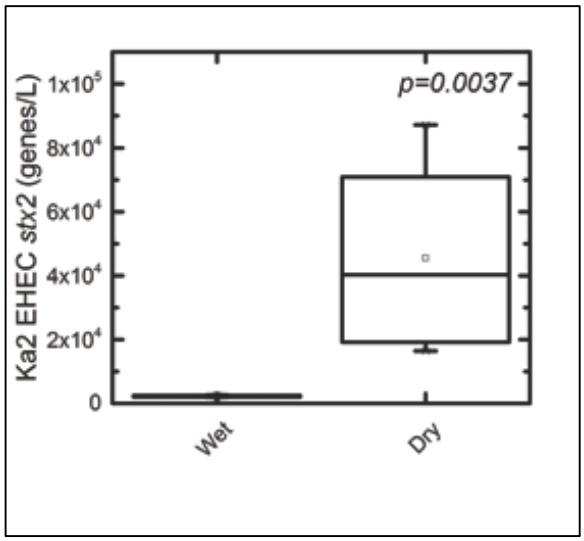

Figure 14. The concentrations of EHEC *stx2* in the public tap suggest that pathogen loading increases during the dry season compared to the wet season, or that there was a breach in the drinking water treatment facility or water distribution system.

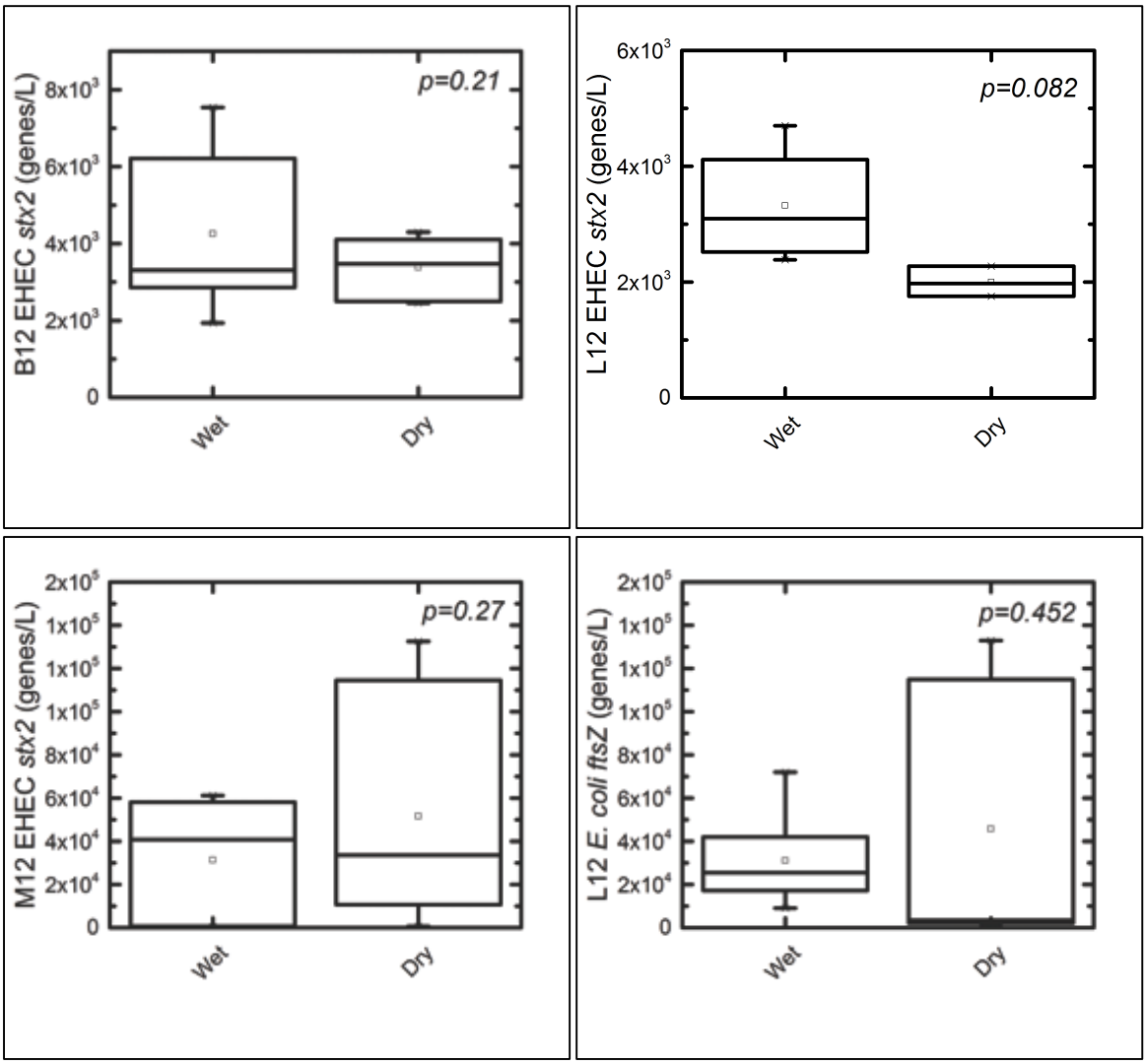

Figure 15. The concentrations of EHEC *stx2* and *E. coli ftsZ* in protected springs suggest that pathogen loading is not significantly influenced by the dry season or wet season.

CHAPTER 4: DISCUSSION

While seasonal variation of waterborne disease incidence is well accepted (62), there has been little research examining the impact of seasonality on waterborne pathogen presence in environmental waters in developing countries. Previous studies provide insight regarding the presence of FIB in environmental waters (63-66), but seasonal analysis of robust microbial water quality measurements is needed, particularly in developing countries where climate change is expected to have the greatest impact on water and food security, and public health (4, 5). Furthermore, high sensitivity and high throughput means of measuring longitudinal microbial water quality in limited resource settings is a knowledge gap that this study was designed to address.

The drainage channels in this study were observed to have a strong relationship to season, with greater target pathogen presence during the wet seasons compared to the dry seasons. Five genes found in drainage channel B3 and one gene found in drainage channel Ka1 showed greater presence in the wet seasons. Also, genes found only as single occurrences in both drainage channels were all during the wet season. Some target genes, such as *E. coli ftsZ* and EHEC *stx2* were not found to be seasonally variable for drainage channel Ka1. This can potentially be explained by the widespread and consistent prevalence of these genes observed in many of the studied water sources (Figure 10). It is likely that these genes were found to be more consistently at drainage channel Ka1 due to greater direct human activity and interaction compared to drainage channel B3. Drainage channel Ka1 was smaller in size and experienced more direct human activity due to a market surrounding the drainage channel. Despite the lack of observed seasonal variation for genes *ftsZ* and *stx2*, the majority of the results show greater occurrence of pathogens in the wet season. These results suggest that both drainage channels become more

contaminated in the wet season as a result of increased runoff into the channel caused by an increase in precipitation.

For surface water samples collected at Lake Victoria, G12, target genes showed inconclusive seasonal variability. Two target genes increased presence in the dry season, and two showed no seasonal variation or only a single occurrence in the wet season. In two Lake Victoria studies, total and fecal coliforms were found to increase in the wet season (67), while the presence of culturable *Vibrio cholerae* was found to increase in the dry season (68). However, these conflicting results could be due to the differences in sampling location. Further investigations of longitudinal microbial water quality in Lake Victoria should include multiple sample collection points along the coast.

While the public tap was observed to have higher positive detection of EHEC *stx2* gene during the dry season, the rest of the positive data points were single occurrences in the wet or dry season resulting in inconclusive seasonal variability. Potential variability at the water treatment plant or in the water distribution system was also considered. A quantitative microbial risk assessment (QMRA) conducted by the water treatment plant found that the risk associated with bacterial contamination in the drinking water distribution system was 2-3 orders of magnitude greater than the risk associated with contamination at the treatment facility (69). This suggests that single occurrences of pathogens in the public tap are more likely due to breaches in the drinking water distribution system, rather than seasonal variability.

Protected springs containing pathogenic microorganisms in wet and dry seasons were not found to be seasonally variable. Overall, the protected springs investigated in this study contained *E. coli ftsZ* and EHEC *stx2* semi-regularly with single occurrences of other pathogens. These data suggest that seasonal variations play little role in altering the water quality of the

protected springs, and hence may not contribute to observed increase in waterborne disease during the wet season.

CHAPTER 5: CONCLUSIONS

In this study, multiple waterborne pathogens were detected in different water sources for a period of seven-months, demonstrating that MFQPCR is a useful technique for long-term biological water quality monitoring, even in resource limited settings. However, there were limitations to the study. A number of MFQPCR assays used for the detection of viruses and bacteria were not used due to poor efficiencies during the MFQPCR plate run. Although all assays achieved approximately 90% efficiency when run with conventional qPCR, some assays did not perform as expected in the final MFQPCR plate. To overcome these limitations, MFQPCR assays should be run with standards only to validate assays prior to sample enumeration. Future studies should also aim to increase sample volume to achieve lower quantification limits. While quantification limits reported were comparable to similar studies, sample processing, reverse transcription, and optional sample clean-up methods all contribute to increasing the lower limit of detection in the original sample. Despite these limitations, the results of this study contributed to the understanding of seasonal effects on microbial water quality of different water source types.

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APPENDIX A: SUPPLEMENTAL DATA

Table 3. Primer and probe sequences for qPCR and MFQPCR assays.

Target Microorganism	Sequence (5'-3')		Ref.
Adenovirus 40/41[b,d]	FP	GGACGCCTCGGAGTACCTGAG	(47)
	RP	ACIGTGGGGTTTCTGAACTTGTT	
	Flu. Pr.	FAM-CTGGTGCAG/ZEN/TTCGCCCGTGCCA-IBFQ	
Enterovirus[a,d]	FP	CCCCTGAATGCGGCTAATC	(48)
	RP	GATTGTCACCATAAGCAGC	
	Flu. Pr.	FAM-CGGAACCGA/ZEN/CTACTTTGGGTGTCCGT-IBFQ	
Human norovirus GI[b,d]	FP	ATGTTCCGCTGGATGCG	(49)
	RP	CCTTAGACGCCATCATCATTTAC	
	Flu. Pr.	FAM-TGTGGACAG/ZEN/GAGAYCGCRATCT-IBFQ	
Human norovirus GII[a,d]	FP	ATGTTCAGRTGGATGAGRTTCTC	(49)
	RP	TCGACGCCATCTTCATTCACA	
	Flu. Pr.	FAM-AGCACGTGG/ZEN/GAGGGCGATCG-IBFQ	
Hepatitis A virus[a,f]	FP	TCACCGCCGTTTGCCTAG	(50)
	RP	GGAGAGCCCTGGAAGAAAG	
	Flu. Pr.	FAM-CCTGAACCTGCAGGAATTAA-NFQ-MGB	
Hepatitis E virus[b,d]	FP	CGGCGGTGGTTTCTGGRGTG	(51)
	RP	GGGCGCTKGGMYTGRTCNCGCCAAGNGGA	
	Flu. Pr.	FAM-CCCCYATAT/ZEN/TCATCCAACCAACCCCTTYGC-IBFQ	

Table 3 (cont). Primer and probe sequences for qPCR and MFQPCR assays.

Target Microorganism	Sequence (5'-3')		Ref.
Rotavirus A[a,d]	FP	ACCATCTACACATGACCCTC	(55)
	RP	GGTCACATAACGCCCC	
	Flu. Pr.	FAM-ATGAGCACA/ZEN/ATAGTTAAAAGCTAACACTGTCAA-IBFQ	
General *E. coli* - *ftsZ*[a,e]	FP	CTGGTGACCAATAAGCAGGTT	(39)
	RP	CATCCCATGCTGCTGGTAG	
	Flu. Pr.	UPL #71	
EHEC - *eaeA*[a,e]	FP	GGCGAATACTGGCGAGACTA	(39)
	RP	GGCGCTCATCATAGTCTTTCTT	
	Flu. Pr.	UPL #28	
EHEC - *stx1*[a,e]	FP	TGTAATGACTGCTGAAGATGTTGAT	(39)
	RP	TCCATGATARTCAGGCAGGA	
	Flu. Pr.	UPL #60	
EHEC - *stx2*[a,e]	FP	TCTGGCGTTAATGGAGTTYAG	(39)
	RP	GTGACAGTGACAAAACGCAGA	
	Flu. Pr.	UPL #126	
Shigella spp. - *ipaH* 7.8[a,e]	FP	TCTGAGAATCCTGACTGAATGG	(39)
	RP	AAGCAATGCCTCGCTCTTC	
	Flu. Pr.	UPL #7	

Table 3 (cont). Primer and probe sequences for qPCR and MFQPCR assays.

Target Microorganism	Sequence (5'-3')		Ref.
Shigella spp. - *ipaH* all[a,e]	FP	AAGGCCTTTTCGATAATGATACC	(39)
	RP	ATTTCGAGGCGGAACATTT	
	Flu. Pr.	UPL #108	
Shigella spp. - v*irA*[a,e]	FP	GGCAATCTCTTCACATCACG	(39)
	RP	TTCGGACATAATTTGGGCATA	
	Flu. Pr.	UPL #6	
Salmonella spp. - *invA*[a,d]	FP	GCATCAATAATACCGGCCTTC	(46)
	RP	ATGGTATGCCCGGTAAACAG	
	Flu. Pr.	FAM-CTCTTTCGT/ZEN/CTGGCATTATCGATCAGTACCA-IBFQ	
Salmonella spp. - *ttrC*[a,e]	FP	GCCTTACAGGCGTTCTTCG	(39)
	RP	ATTTTTGGCAGCCTTACCG	
	Flu. Pr.	UPL #149	
Camplyobacter jejuni - *ciaB*[a,e]	FP	GCGTTTTGTGAAAAAGATGAAGATAG	(39)
	RP	GGTGATTTTACTTTCATCCAAGC	
	Flu. Pr.	UPL #137	
Legionella pneumophila - *mip*[a,e]	FP	GGATAAGTTGTCTTATAGCATTGGTG	(39)
	RP	CCGGATTAACATCTATGCCTTG	
	Flu. Pr.	UPL #60	

Table 3 (cont). Primer and probe sequences for qPCR and MFQPCR assays.

Target Microorganism	Sequence (5'-3')		Ref.
Pseudogulbenkiania sp. NH8B[b,e]	FP	CAGGCCGTGAAGTCAAGC	(39, 53)
	RP	GAGGCGATGTGGATGGTC	
	Flu. Pr.	UPL #56	
Vibrio Cholerae - ctxA[a,d]	FP	TTTGTTAGGCACGATGATGGAT	(39)
	RP	ACCAGACAATATAGTTTGACCCACTAAG	
	Flu. Pr.	FAM-TGTTTCCAC/ZEN/CTCAATTAGTTTGAGAAGTGCCC-IBFQ	
Cryptosporidium[b,f]	FP	GGGTTGTATTTATTAGATAAAGAACCA	(54)
	RP	AGGCCAATACCCTACCGTCT	
	Flu. Pr.	FAM-TGACATATCATTCAAGTTTCTGAC-NFQ-MGB	

Table 4. MFQPCR specifications for standards used in final quantification.

Assay	LLQ	ULQ	Slope	r^2	Efficiency	Plate
Enterovirus	3	3×10^6	-3.45	0.96	95	0.45
E. coli ftsZ	3	3×10^6	-3.32	0.98	100	0.45
EHEC *eaeA*	30	3×10^6	-3.61	0.99	89	1.6
EHEC *stx1*	3	3×10^6	-3.36	0.99	99	1.6
EHEC *stx1*	3	3×10^6	-3.25	0.94	103	0.45
EHEC *stx2*	3	3×10^6	-3.42	0.96	96	1.6
Shigella spp. *ipaH 7.8*	3	3×10^6	-3.44	0.96	95	1.6
Shigella spp. *ipaH all*	3	3×10^6	-3.21	0.97	104	1.6
Salmonella spp. *invA*	3	3×10^6	-3.35	0.95	99	0.45
Salmonella spp. *ttrC*	3	3×10^6	-3.252	0.98	103	1.6
Salmonella spp. *ttrC*	3	3×10^6	-3.16	0.94	107	0.45
Vibrio cholerae ctxA	3	3×10^6	-3.37	0.94	98	0.45