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I, Brian D Zimmerman, hereby submit this original work as part of the requirements for the degree of Master of Science in Environmental Science.

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Human Mitochondrial DNA and Endogenous Bacterial Surrogates for Risk Assessment of Graywater Reuse

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Human Mitochondrial DNA and Endogenous Bacterial Surrogates for Risk Assessment of Graywater Reuse

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Abstract

Groundwater aquifers and surface waters currently used as drinking water and irrigation sources are in danger of over exploitation, leading to potable water scarcity in many regions of the world. On-site treatment and reuse of recycled wastewaters such as graywater for non-potable purposes has the ability to enhance water sustainability by alleviating demands on potable water supplies, which is particularly valuable in arid regions or in times of severe draught. However, given the inevitable downstream human contact, graywater represents a waterborne pathogen transmission and amplification pathway if human exposure to reused water is practiced without adequate treatment. Enteric pathogens are currently thought to be one of the most significant public health risks to water reuse. (1) Thus, previous studies sought to predict enteric pathogen presence in graywater through the use of fecal indicator bacteria (FIB) to indicate human fecal contamination and possible pathogen presence. However, FIB are known to grow in stored graywater, (2) do not correlate well with pathogens, (3) and may not accurately predict risks from pathogens transmitted via respiratory/oral and dermal pathways. (4) Therefore, new metrics to measure and predict microbial risk in graywater recycling systems is necessary for advancement of these systems.

Due to potential pathogen presence, it is recommended that graywater undergo biological treatment and disinfection prior to reuse if downstream human contact is expected. U.S. graywater guidelines (2012) suggest using fecal coliforms of 0/100mL as the most conservative disinfection surrogate for reuse. (5) However, their quantities at different stages of treatment may vary due to re-growth, (6) causing inaccurate readings of the microbial log removal. Therefore, there is also a need for better microbial surrogates that can be used during graywater treatment to indicate process performance and pathogen reduction. Technologies such as high

throughput DNA sequencing and quantitative polymerase chain reaction (qPCR) can assist with identifying novel surrogates potentially suited to evaluate pathogen removal in these systems.

In this investigation, we utilize high throughput pyrosequencing and qPCR to identify and quantify select bacterial and human surrogates and pathogens in industrial laundry graywater sourced from the University of Cincinnati's athletic facility. Pyrosequencing and qPCR revealed that laundry water microbiota was dominated by the skin-associated bacteria *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* (6.5, 5.7, 5.4 log₁₀ copies/100mL respectively). While human mitochondrial DNA (HmtDNA) was less abundant (2.8 log₁₀ copies/100mL) it showed strong positive correlations with these three genera ($r \geq 0.45$, $P \leq 0.002$) as well as the opportunistic pathogen *Staphylococcus aureus* ($r = 0.54$, $P = 3.2 \times 10^{-4}$). Further, HmtDNA closely followed a first order exponential decay model ($R^2 = 0.98$), remaining detectable in stored laundry graywater for up to six days at 20°C. Based on consistency, abundance, and persistence in graywater, this research identifies HmtDNA and skin-associated bacteria such as total *Staphylococcus* as potential molecular surrogates for measuring microbial log removal in future graywater treatment evaluations.

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List of Acronyms

μS: micro seconds
260/280: 260nm vs. 280nm ratio as measure of DNA purity
6F – 6-Carboxyfluorescein
Bac: Total *Bacteroides* spp.
BC: Building control
BOD: Biological oxygen demand
Bp: base pair
Cm: Centimeter
CO₂: Carbon dioxide
COD: Chemical oxygen demand
Coryne: *Corynebacterium*
Cq/Ct: Cycle of quantification/threshold during PCR
CSU: Colorado State University
CV: Central Valley aquifer (California)
DO: dissolved oxygen
E. coli: *Escherichia coli*
Entero: *Enterococcus* spp.
ET: Equalization tank
F: Forward (primer)
FIB: Fecal indicator bacteria
GC content: Guanine/Cytosine content of DNA
GW: Graywater
HBac: Human-specific *Bacteroides*
HmtDNA: Human mitochondrial deoxyribose nucleic acid
HP: High plains aquifer (central U.S.)
HPC: Heterotrophic plate count
KCl: Potassium chloride
L: Liter
LLOQ: Lower limit of quantification
Mg/L: Milligrams per liter
MGB – Minor groove binding
MgCl₂: Magnesium chloride
mL: Milliliter
mm: Millimeter
MRSA: Methicillin-resistant *Staphylococcus aureus*
MST: Microbial source tracking
mv: Millivolt
ng/μL: Nanogram per microliter
Nm: nanometer
NR: Not reported
NTC: No template control
NTU: Neophilactic turbidity unit
O₂: Oxygen
PBNQ: Present but not quantifiable

PC: Principal coordinate
PCoA: Principal coordinate analysis
Ppt: Parts per trillion
Propi: *Propionibacterium*
Pse: *Pseudomonas* spp.
PW: Potable water
qPCR: quantitative real-time polymerase chain reaction
R: Reverse (primer)
rRNA: ribosomal ribose nucleic acid
SH: Shower graywater
StaphA: *Staphylococcus aureus*
TA – Tetramethyl rhodamine (TAMRA)
TKN: Total Kjeldahl Nitrogen
TSS: Total suspended solids
TStaph/Staph: Total *Staphylococcus*
U.S.: United States
UC: University of Cincinnati
VSS: Volatile suspended solids

Chapter1: Introduction

Background

Groundwater aquifers and surface waters currently used as drinking water and irrigation sources are in danger of over exploitation, leading to potable water scarcity in many regions of the world. Human-induced changes that alter the natural water cycle such as “gray” infrastructure and increasing population densities has led to groundwater withdrawal exceeding recharge rates and escalated contamination. (7) The expected population changes for the United States (U.S.) through 2030 can be seen in **Figure 1**, with color representing population change and the 3-D bars representing current population.

Figure 1. Population growth trends for the US. (8)

In addition, changing climatic patterns favoring prolonged drought periods in some heavily populated areas are expected to exacerbate water scarcity effects. (8) **Figure 2** depicts projected

annual precipitation changes for the US based on 112 climate model projections.

Figure 2. Annual precipitation changes (cm/year) in the US based on 112 30-year climate projections. Adapted from Brekke et al., 2009. (8)

If figures 1 and 2 were overlaid, the result would be increased population with decreasing precipitation in a majority of the central and western U.S., a region that relies heavily on groundwater to support potable and irrigation water needs. The groundwater recharge rates for two important aquifers, the High Plains (HP, central U.S.) and Central Valley (CV, California), are depicted in **Figure 3**. In this study, the authors concluded that increased water withdrawal in these aquifers will exceed future recharge rates, rendering them unable to support water needs within 30 years. (9)

U.S. states such as Tennessee and Georgia have filed law suits to adjust state borders to gain access rights to potable water supplies. (10) Furthermore, US water allocations are based on 100-year old water rights, which did not factor in today's climate and population challenges, and therefore, when fully utilized, do not meet expected demands. (11) For instance, water contracts were developed in the southwestern U.S. to divvy up water rights; In 2013, the U.S.

Bureau of Reclamation implemented a 20% reduction in previously guaranteed water that was contracted to farmers in southern Arizona, (12) demonstrating the serenity of the water situation in certain regions of the U.S. Thus, new approaches to potable water usage and management, such as water reuse, have been proposed and discussed amongst authorities.

Figure 3. (A) Measured groundwater changes from ~1950 to 2007 in the HP aquifer. (B) Simulated groundwater level changes from ~1860 to 1961 in the CV aquifer. Groundwater basins include the Sacramento, Delta/East sides, San Joaquin, and Tulare. (9)

Water Reuse

In general, water reuse is the practice of treating and recycling used residential or industrial wastewater for non-potable applications, with intent of reducing demands on potable supplies in arid regions and/or in times of water shortage. (13) However, direct potable reuse (i.e. treating wastewaters for potable purposes) is now being planned at several locations in Texas and California. If water reuse is to be a feasible water management strategy given economic considerations and full life-cycle costing, one potential strategy is to include source-separation of wastewater components to realize the benefits of each resource ('waste') stream. Separation of toilet waste (blackwater) from other household water (graywater) can capture the added benefits of nutrient recovery from concentrated fecal and urine streams in blackwater, while lowering treatment and energy costs as well as health risks associated with reusing treated graywater, resulting in reduced demands on potable water supplies. (14) However, due to the potential health risks of reusing domestic wastewaters, such as graywater, there is no federal regulatory framework in the U.S. to govern reuse systems; only guidance documents exist. (15) In addition to the potential presence of pathogens, there is a limitation of general biological knowledge about this matrix, complicating the design of treatment systems that are necessary to remove pathogens to safe levels for reuse. Thus, this study's focus was to gain a better understanding of the microbial composition of graywater and to identify potential microbial metrics that could be used as a surrogate to evaluate pathogen removal in future graywater treatment studies.

Graywater Quality

Graywater is defined as any type of water used within a home or residential setting that does not include toilet flush water, i.e. shower and bath, dishwashing, laundry, and sink water,

(1) while blackwater is limited to toilet waste only. (14) On-site treatment and reuse of graywater appears to be an economically attractive element in municipal water management, when compared to blackwater, (16) for use in non-potable applications such as irrigation, toilet flushing, and vehicle washing. This is because graywater is expected to be less polluted than blackwater, and in theory, would require less treatment for safe non-potable reuse. However, previous graywater composition studies have shown significant but varying levels of microbial parameters such as total coliforms, fecal coliforms, heterotrophic plate counts (HPC), bacterial pathogens, parasitic protozoa, and enteric viruses, as well as chemical parameters such as COD, BOD, TSS, VSS, and electrical conductivity. (1, 2, 16, 17) A list of reported graywater pathogens is presented in **Table 1** while a list of reported chemical characteristics is listed in **Table 2**. As a result, graywater quality has been concluded to lie somewhere between raw wastewater and secondary effluent, (18) and has even been broken into classes such as ‘light’ and ‘dark’ graywater to exclude food wastes from kitchen sinks and laundry. (19) However, the presence of enteric pathogens indicates that graywater reuse may pose a risk to human health if reused without adequate treatment. (20)

Table 1. Pathogens in graywater (1, 2, 17-19, 21)	Conc. Log ₁₀ /100mL ¹ .
<i>Pseudomonas aeruginosa</i>	3.5-4.3
<i>Staphylococcus aureus</i>	4.0
<i>Legionella pneumophila</i>	2.2-2.9
<i>Salmonella</i> spp.	PBNQ
<i>Cryptosporidium</i> spp. ² .	0-8.3 ² .
<i>Giardia</i> spp. ² .	0-7.9 ² .
Norovirus	PBNQ
Rotavirus	PBNQ
Enterovirus	PBNQ

1. PBNQ: present but not quantifiable

2. Conc. log₁₀/100L

Table 2. Characteristics of ‘light’ graywater from various studies adapted from Birks et al., 2007(19)

Reference	Laine, 2001 (22)	Birks et al. 2004; (23)Smith et al. 2001 (24)	Surendran and Wheatley 1998 (25)	Rose et al. 1990 (1)	Christova-Boal et al. 1996 (26)
Graywater type	B, S, W	W	B, S, W	B, S, W, L	S, B
BOD (mg/l)	129-155	5-142	216-252	NR	76-200
COD (mg/l)	367-587	21-355	424-433	NR	NR
SS (mg/l)	58-153	7-102	40-76	NR	48-120
Turbidity (NTU)	60-164	NR	57	20-140	113
pH	7.3-7.5	NR	7.7	5-7	6.4-8.1
Ammonia, as N (mg/l)	NR	NR	0.5-1.6	0.15-3.2	<0.1-15
TKN, as N (mg/l)	NR	0-23.3	NR	0.6-5.2	4.6-20
Total phosphorous (mg/l)	0.3-0.4	NR	1.6-45.5	4-35	0.11-1.8

NR: Not reported in study; B: bath; S: shower; W: washbasin; L: laundry

Human Fecal Contamination in Graywater

In general recreational and multi-use waters, human fecal contamination is currently thought to represent the highest risk to human health due to its potential highly concentration of enteric pathogens transmitted via the fecal-oral route. (4) Thus, organisms such as total coliforms, fecal coliforms, *E. coli*, enterococci, sulphite-reducing clostridia/*Clostridium perfringens*, and human-specific *Bacteroides*, which have presumably been demonstrated (or thought) to be of fecal origin [deemed fecal indicator bacteria (FIB)], have been used to monitor for human fecal contamination in water applications. (3, 27) A list of some of these indicator bacteria in graywater is displayed in **Table 3**.

Table 3. Indicator bacteria in graywater streams adapted from Winward et al. 2009 (4)

Graywater Source	Total Coliforms (log ₁₀ /100mL)	Fecal Coliforms (log ₁₀ /100mL)	E. coli (log ₁₀ /100mL)	Enterococci (log ₁₀ /100mL)	Heterotrophic bacteria (log ₁₀ /1mL)
Wash basin	4.7 - 5.8	1.5 - 3.5	3.8	>2.3	>5.5
Shower	3.0 - 5.0	1.0 - 6.6	2.8 - 3.2	1.5 - 3.3	5.0 - 8.4
Bath	1.7 - 4.4	6.6	1.9 - 4.3	1.0 - 1.6	NR
Light gray	2.7 - 7.4	1.0 - 5.7	0.5 - 4.4	1.9 - 3.4	5.0 - 7.4
Laundry	1.7 - 5.8	1.4 - 6.6	NR	1.4 - 3.4	7.6 - 8.3
Kitchen sink	NR	6.1	NR	NR	NR
Dishwasher	NR	4.8	NR	NR	NR
Dark gray	7.2 - 8.8	4.9 - 7.9	2.0 - 6.0	2.4 - 4.6	NR

NR: Not reported

While human FIB have routinely appeared in low levels in graywater, pathogen presence has been more sporadic, indicating the major risk contribution is limited to single, infected individuals. (1) However, the challenges of isolating and quantifying limited pathogen excretion events, coupled with the diversity of potential pathogens present renders single pathogen monitoring programs uneconomical. (27) Hence, the commonly accepted approach is to monitor for traditional FIB that are presumably indicative of human fecal contamination and thereby represent the potential presence of human pathogens, under the assumption that the larger the pool of contributors, the higher the risk of an infected source contribution. (3) However, indicators such as total coliforms, fecal coliforms, and *E. coli* have been shown to exhibit growth in the environment and in graywater, (2, 6) representing increased rates of false positive detection of human fecal pollution. In addition, fecal indicators are always excreted in feces whether or not the individual is shedding pathogens, suggesting that fecal indicators and pathogens are unlikely to vary quantitatively. (3) Furthermore, pathogens are much more sporadic in their occurrence, and pathogen prevalence and fecal loads are highly variable depending on the source of graywater in the household. (19) In fact, a 2012 Australian study reported no correlation between *E. coli* indicators in laundry water with the presence of

enterovirus, norovirus, or rotavirus, (17) demonstrating a need for more appropriate biological indicators or new ways of assessing microbial graywater quality.

Some water-stressed regions, such as most of Australia's capital cities and the south and western U.S. have already started to reuse graywater for various purposes, even without treatment. However, reuse of human-contacted graywater raises potential health concerns due to the transfer of waterborne pathogens, especially since no indicator or set of markers has been demonstrated to accurately predict human health risk. (1, 28) Even though some regions have already adopted reuse approaches, the lack of microbial information has hindered reuse acceptance leading to individualized (State-specific in U.S.) regulatory approaches. (29) Thus, there is a knowledge gap in the microbial composition of graywater relating to health risk and treatment requirements necessary to safely reuse graywater.

Surrogate Approach

One accepted approach to assess general wastewater treatment performance for biological pathogen removal/inactivation is by using a surrogate to represent microbial treatment removal efficacy. (30, 31) This approach typically uses a non-pathogenic organism or chemical as a surrogate to represent a pathogen or group of pathogens based on factors such as size, structure, abundance, persistence, and behavior relative to a particular treatment. (31, 32) Spores of non-pathogenic *Bacillus* have previously been used as surrogates for anthrax (31) and *Clostridium perfringens* as surrogates for protozoan oocysts and enterovirus. (33) Bacteriophages of *E. coli* (coliphages – F-specific and somatic) (33) and *Bacteroides*, (34) (GB-124), (35) have also been used or proposed for virus inactivation/removal studies. If not in the desired abundance of the matrix itself, these organisms are typically cultured in a lab and spiked into the treatment matrix once demonstrated (or theorized) to behave similarly to pathogens of interest. However,

effective graywater management as part of an overall municipal water system likely requires a decentralized approach, making the use of externally spiked surrogates to verify performance uneconomical. Furthermore, the decentralized infrastructure of graywater reuse requires low operator upkeep since public recognition of high maintenance systems is unlikely to warrant acceptance, producing a need to identify endogenous microbial markers or surrogates of water quality that could be used to evaluate risk and/or on-site graywater treatment performance, eliminating the need for routine surrogate spiking. (8)

High Throughput Sequencing

DNA sequencing technologies have been used in microbiology since the 1970's to obtain viral, prokaryotic and eukaryotic genomes. However, only recently have high throughput sequencing technologies become cost effective and reliable to enable the gathering of genetic information to provide insight and understanding of complex environmental microbiomes. The 454 Pyrosequencing platform by Roche Inc., the Mi-Seq and Hi-Seq platforms by Illumina, and the Ion Torrent by Life Technologies represent three high throughput sequencing technologies currently on the market today. These systems are proprietary inventions that all operate based off of slightly different principles. For example, Pyrosequencing is based on the synthesis-by-sequencing principle and detects the pyrophosphate released when synthesizing DNA (36) as depicted in **Figure 4**, whereas the Ion Torrent uses a semiconductor to detect the H^+ ion being released when nucleotides are added during synthesis. (37) A review by Liu et al., 2012 summarizes some of the newest developments and principles of DNA sequencing technologies on the market as of 2012. (38)

Figure 4. Schematic of Pyrosequencing adapted from Fakruddin et al., 2012 (36)

These technologies have many uses in DNA sequencing but for the purposes herein, they have the ability to provide previously unattainable genetic information about the bacterial composition from environmental samples by sequencing of the 16S rRNA gene, which is common to all *Bacteria* and *Archaea*. The 16S rRNA gene is approximately 1500 base pairs (bp) in length (*E. coli* numbering) and consists of 9 variable regions that genetically differ between bacterial taxa. These variable regions are interspersed with conserved regions that permit primer annealing for amplification of the hypervariable region between the primers, thereby allowing downstream identification to various taxonomy levels for certain bacteria.

Bacterial sequencing has been successfully utilized to characterize the bacterial populations in various matrices such as groundwater, (39) surface water, (40) wastewater, (41) drinking water, (42) and biofilms, (43) allowing for significant advancements in the understanding of the microbiome in these environments. However, bacterial sequencing of graywater has yet to be addressed, hindering understanding of the bacterial composition of this

complex matrix. To meet this need, unidirectional 454 Pyrosequencing of DNA extracted from laundry graywater and a shower graywater recycling system was employed to identify abundant bacteria that could be targeted for more specific molecular quantification technologies such as quantitative polymerase chain reaction (qPCR).

Quantitative Polymerase Chain Reaction (qPCR)

qPCR uses the 5' to 3' nuclease activity of an enzyme that is often isolated from *Thermus aquaticus*, also known as *Taq*, (44) to amplify DNA to detectable levels. Reaction components typically consist of primers, nucleotides, salts such as KCl and MgCl₂, a buffer such as 10mM Tris, pH 8.4, the polymerase, water and the DNA template. (45) qPCR is performed in a thermocycler and consists of three steps: 1) DNA template denaturing, 2) annealing of the primers, and 3) and extension where the DNA is copied. During step 1, the reaction is heated to 90-96°C to denature the DNA template. During step 2, the forward and reverse primers (set of nucleotides that corresponds to the binding site of the DNA template) then bind to the complementary DNA strand, also known as the annealing phase, which takes place between 50-70°C. During step 3, (the final phase) commonly called extension, (usually between 68-75°C), the *Taq* polymerase recognizes the primer binding sites and proceeds to amplify the template DNA strand exponentially with each cycle, making 2^x copies where x = the number of cycles under perfect amplification reaction conditions. The reaction undergoes anywhere from 25 to 55 cycles depending on the application, exponentially increasing until a plateau phase is reached when reaction components necessary for amplification are limiting. During a specifically designed qPCR approach called Taqman probes (**Figure 5**), a strand of DNA (called a probe) that is located within the primer binding location is labeled with a fluorescent reporter and quencher. (44) When the reporting and quenching dyes are intact, very little fluorescence is

observed. However, when the enzyme proceeds with amplification, the probe is cleaved and produces a measured fluorogenic response. Once the fluorogenic response is observed beyond background levels, a C_t or C_q (cycle threshold or cycle of quantification), which is the cycle at which that fluorescence is observed above background, can be compared to a standard curve for absolute quantification of the original sample. qPCR assays can be made as specific for a particular species or can be made broad to encompass an entire genus, allowing for detection of as little as between 1 to 10 gene copies per reaction mixture, which is approximately 1-2 logs more sensitive than traditional culture techniques and shortens quantification time from days to hours. However, the limitation with qPCR is that an organisms DNA can still be detected even if the organism itself is incapable of replication or in a stressed state, deemed viable but not culturable. (46) Therefore, qPCR can detect an organisms' DNA but that organism may not be capable of infecting a host.

Figure 5. Taqman qPCR (47)

To date, literature searches have only revealed the use of qPCR (or conventional PCR) for a very small number of graywater studies, with nearly all of those studies targeting pathogens as opposed to indicators or surrogates. However, virtually all of these studies concluded that enteric pathogens may be present in graywater, but proved no useful relationships for predicting human health risk. (17) Therefore, I have utilized a Taqman qPCR approach to quantify several fecal or potential graywater indicator bacteria, surrogates and/or human markers in laundry graywater, since it is the most abundant by volume. (48) Using qPCR, I targeted the fecal indicators: *E. coli*, *Enterococcus* spp., and human-specific *Bacteroides*; for potential graywater surrogates: universal *Bacteroides* spp., *Pseudomonas* spp., *Staphylococcus* spp., *Propionibacterium*, *Corynebacterium*, and human mitochondrial DNA (HmtDNA); and for pathogens: Adenovirus (enteric, conventional PCR) and *S. aureus* (dermal). *E. coli*, *Enterococcus* spp., and human-specific *Bacteroides*, were chosen based on their fecal origin to gauge the fecal contamination in laundry graywater since previous studies reported high levels of fecal contamination. (6) Adenovirus was targeted as an enteric pathogen since they are considered one of the groups of greatest human health risk and one of the most numerous enteric virus groups in wastewater, (49, 50) *Pseudomonas* spp. was chosen for its ability to be present in high abundances in many matrices and *S. aureus* based on its opportunistic pathogenicity originating from human skin. The remaining skin targets were chosen based on abundance identified by high throughput bacterial sequencing, except for HmtDNA which was chosen for reasons discussed below.

Human Mitochondrial DNA

HmtDNA was chosen as a potential surrogate because it is a robust, species-specific 16,569 bp circular genome found in the epithelial cells of human stool, blood, and saliva on the

order of hundreds to thousands of copies per cell. (51-53) Due to these characteristics, it has recently been used to differentiate and identify non-point sources of fecal pollution, known generically as microbial source tracking (MST), from various mammalian species in surface and wastewaters. (54-61) Due to the human specificity of HmtDNA, high copy number per cell, inability to self-replicate outside the host organism, and superior persistence when compared to larger chromosomal DNA, this investigation sought to evaluate its use as a human marker in graywater and to determine if it could be used as a surrogate to gauge on-site graywater treatment performance and possibly pathogen presence. In addition, should the persistence of HmtDNA in graywater be predictable, it could also assist in identification of graywater age and potential increased risk to reuse.

Research Goals

After over one hundred years of using centralized sewage infrastructure for removal of waterborne pathogens, (62) revisiting separate wastewater streams, such as graywater, for water conservation is practical. However, there is a paucity of information on microbial characteristics that may be indicative of human health risk. Therefore, there were two major aims of this study:

1. employ bacterial 16S rRNA gene pyrosequencing to identify the metagenomic profile of bacteria in two distinct sources of graywater: laundry water sourced from an industrial washing machine and from a graywater recycling system in a college dormitory
2. use qPCR to quantify traditional fecal indicator bacteria (FIB), select bacteria (based on sequencing results) and human mitochondrial (HmtDNA) surrogates, and fecal and skin pathogens in laundry graywater

to gather more information on human health risk and to identify candidate surrogates for evaluating on-site graywater treatment performance

Laundry water was chosen specifically for quantification of select targets since it was a readily available source and comprises the largest volume of water within a household (**Figure 6**), (48) allowing for the highest potable water saving capabilities if reuse is applied. To the authors' knowledge, this study was the most extensive study to use a high-throughput DNA sequencing and a qPCR-based approach to identify and quantify potential bacterial and human surrogates (such as HmtDNA), several FIB, and both skin and fecal pathogens in graywater, and explore their relationships. This research specifically evaluates the use of HmtDNA and other skin-associated bacteria such as total *Staphylococcus* as molecular surrogates for assessing microbial risk and pathogen removal during graywater treatment, and highlights the use of modern high throughput sequencing and molecular quantification technologies for general use in graywater risk assessment.

Figure 6. Residential graywater use by volume in U.S. (48)

Chapter 2: Pyrosequencing of Graywater Samples

Portions of Chapter 2 are expected to be submitted to *Applied & Environmental Microbiology*

Introduction

Graywater (GW), consisting of used water from shower, bath, laundry, sink, and kitchen sources is an attractive water reuse alternative to alleviate potable water burdens, given its lower microbiological and nutrient levels relative to blackwater. (4, 63) On-site collection, treatment, and reuse of source-separated GW also may enhance water systems sustainability by facilitating energy and nutrient recovery from concentrated fecal and urine streams. (14) However, GW reuse acceptance has been hindered due to the detection of human pathogens found in previous GW investigations. (4, 17, 19, 64) Due to the fecal-oral route of water, human enteric pathogens have been considered the greatest risk to human health during reuse, and have been the center piece of previous investigations. (65-67)

Since fecal indicator counts have been consistently abundant in previous studies, it has been inferred that graywater contains significant amounts of human fecal contamination. (2) However, fecal indicators have been shown to grow in graywater, (6) and their relationships to pathogens have been found unreliable at predicting risk. (17) Due to these limitations with the use of fecal indicators and pathogens, our understanding of GW microbiology is inadequate. Hence, there is a need to further differentiate the microbiological relationships and risk present in GW if proper design and acceptance of reuse strategies are to be adopted.

High-throughput sequencing and bioinformatic analysis has recently become a cost-effective tool for defining spatial and temporal patterns in the occurrence of a broad range of microorganisms, especially bacteria, within environmental samples leading to an improved understanding of the factors regulating microbial community structure. Application of this approach to GW would broaden our understanding of bacterial composition in GW beyond pathogen occurrence and could provide information on GW ecological interactions. Additionally,

common, abundant bacterial constituents of GW may be more useful than fecal indicators as surrogates for measuring treatment efficacy. The high abundance of the dominant members could provide a wide dynamic range to estimate log removal rates of different GW reuse treatment approaches, which, after correlation to the removal rates of pathogens in controlled spiking experiments, could be used as endogenous metrics of treatment process efficacy.

Bacterial 16S Ribotyping has recently been successfully utilized to characterize a variety of water matrices such as groundwater, (39) surface water, (40) wastewater, (41) drinking water, (42) and biofilms, (43) allowing for significant advancements in the understanding of the microbiome in these environments. Applying this approach to graywater is a necessary first step to defining important GW constituents and factors affecting their presence. Hence, we identified the bacterial metagenomic profiles of two distinct sources of graywater: shower water from a university dormitory recycling system and laundry water from an athletic facility industrial washing machine. Laundry samples were collected directly from the outlet of the washing machine, prior to drainage trough contact, while shower samples were taken from various points in the collection system (after passage through 1.5-20.7m of shower drain collection pipes, and after storage in an equalization tank used to store shower water before treatment and reuse) to evaluate the potential influence of ecological change on bacteria assemblages within the GW collection system. In sum, 24 laundry samples, 18 shower samples, and 6 tank samples were taken for this study. Abundance analysis, heat maps, and principle coordinate analysis (PCoA) were used to assist with identifying relationships amongst graywater samples.

Methods and Materials

Laundry Sampling

The University of Cincinnati in Cincinnati, Ohio, United States, uses two washing machines (Pellermil Milnor Corporation models 36026 V5J and 30022 V6J) to wash the University's 500+ student athletes' sportswear and towels. One washing machine is dedicated mostly to clothing attire while a smaller washer services towels. The laundry facility has a collection bin in which all athletes place their laundry onto a loop to be washed by the equipment room staff. The large clothing washer has a 0.43m^3 capacity and discharges roughly 250 liters per cycle. The washer is run daily using the following conditions: a 3-minute rinse (21°C), 6-minute wash (29°C), 12-minute wash (29°C), followed by two 2-minute rinse cycles at (27°C and 21°C) discharging approximately 250 liters of water per cycle, and a final spin-only extract. The same concentration of detergent (Tide 2X concentrate, Procter & Gamble Co.) is automatically injected for each wash cycle. All discharges are pooled into a collection basin which connects to the sewer drain. One-liter grab samples were taken from both the pre-rinse ($n = 20$, no detergent) and first wash ($n = 20$, detergent included) in sterile 1-L Nalgene bottles halfway through the approximate 40-second discharge period from February to May, 2013. For this portion of the study, 12 pre-rinse and 12 wash samples (6 of each from winter and spring) were sequenced. Collection of the first two cycles was based on the expectation that a majority of microbes from clothing would be removed during these first two cycle discharges, (16) thereby representing the highest predicted biological presence and highest microbial risk associated with graywater reuse. Samples were placed on ice and immediately transported to the lab for concentration and nucleic acid extraction within two hours.

Sample Concentration and DNA Extraction

Samples were vacuum filtered through 47 mm diameter, 0.45 µm mixed cellulose ester (nitrocellulose) membranes (Pall Corporation, Inc.). Filtrate volume ranged from 70-380 mL to ensure adequate DNA concentrations. Filter blanks using 100 mL of double-distilled water were used to monitor for extraneous DNA contamination. Filters were removed from the filter cone apparatus using sterile forceps and placed into 47 mm Petri dishes (Pall Corp., Inc.) and processed immediately or stored at -70°C until DNA extraction. DNA was extracted using the PowerWater DNA Extraction Kit (MoBio Labs, Inc.) according to the manufacturer's instructions. DNA extracts were quantified using a ND-1000 Nanodrop spectrophotometer (NanoDrop Technology, Wilmington, DE) and stored at -70°C. **Table 4** shows the volume of laundry water filtered and the concentration of extracted DNA in all laundry samples.

CSU GW Recycling Sampling

To assess the bacterial composition of graywater in the Colorado State University dormitory water recycling system, water samples were taken from a graywater system that collects shower and sink hand wash water (SH), and a shower water equalization tank (ET). Graywater SH samples were obtained from a dormitory located at Colorado State University (CSU) in Ft. Collins, Colorado and an ET in its basement. SH samples were collected in February (2 days), March (4 days), April (1 day), May (5 days), and December (6 days) of 2012 and April (3 days) of 2013. Water samples were collected from the ET in May (3 days) 2012 and April (3 days) of 2013. Building control (BC) samples were obtained in May (3 samples) 2013 to assess the contribution of drain pipe bacteria. In addition, a single water sample was collected from a shower head, deemed potable water (PW). In sum, 24 LA, 21 SH, and 6 ET, 3 BC and one PW sample were collected for this study.

Table 4. Sample Concentration Information

Sample number	Sample Date	Cycle	DNA Yield (ng/ μ L)	260/280 ratio	mL filtered
1	2/1/2013	pre-rinse	5.2	1.44	250
2		first wash	4.9	1.46	100
3		first wash	5.6	1.50	250
4	2/11/2013	pre-rinse	4.4	1.35	250
5		first wash	2.8	1.47	100
6		pre-rinse	5.4	1.19	250
7		first wash	5.6	1.34	100
8	2/13/2013	pre-rinse	6.6	1.03	250
9		first wash	3.9	1.18	100
10	2/14/2013	pre-rinse	5.6	1.44	380
11		first wash	4.7	1.49	300
12		pre-rinse	5.3	1.77	250
13		first wash	5.5	1.77	200
14	2/18/2013	pre-rinse	5.3	1.45	200
15		first wash	4.6	1.44	100
16	2/20/2013	pre-rinse	7.3	1.52	250
17		first wash	8.4	1.61	250
18		first wash	2.5	1.39	210
19	2/25/2013	pre-rinse	8.7	1.58	250
20	3/4/2013	pre-rinse	6.8	1.61	100
21		first wash	3.5	1.73	250
22		pre-rinse	3.5	2.67	210
23	3/18/2013	pre-rinse	3.2	3.51	100
24		first wash	2.2	3.07	250
25		pre-rinse	3.8	1.95	100
26		first wash	3.3	2.29	200
27	3/25/2013	pre-rinse	2.6	1.61	70
28		first wash	4.5	1.18	225
29	5/13/2013	pre-rinse	7.7	1.8	250
30		first wash	2.6	2.37	200
31	5/20/2013	pre-rinse	12.8	1.5	200
32		first wash	5.7	1.3	150
33		pre-rinse	11.4	1.25	200
34		first wash	8.5	1.3	150
35	5/23/2013	pre-rinse	5	2.5	250
36		first wash	4.7	2.02	150
37	5/28/2013	pre-rinse	13.4	1.86	200
38		first wash	3.3	1.77	150
39		pre-rinse	2	1.81	200
40		first wash	5.8	1.6	200

Shower and hand wash water was sampled from the restrooms in 14 residence hall rooms with a maximum occupancy of 28 persons. The SH water flowed into a GW collection system and then to the 250-gallon ET located in the basement of the residence hall. SH samples were collected by opening a valve just prior to entry into the ET. For each SH sample, first a minimum of 50 L of GW was collected in a 132-L compositing tank, prior to ET, to obtain a representative mixed sample. Then, triplicate 100-mL sub-samples were taken from 1-L bottles filled with GW collected by opening a valve on the 35-gallon compositing tank. Each GW sub-sample was filtered through a 0.45 μ m membrane, and bacterial DNA was extracted using the PowerWater[®] DNA Isolation Kit (MoBio Laboratories) according to the manufacturer's instructions. Triplicate DNA extractions were then pooled by volume prior to analysis. For each ET sample, water was collected by opening a valve just prior to entry into a graywater treatment system. Then samples (100 mL) were filtered and DNA was extracted as described previously. Decreased filtrate volume for ET samples was due to filter clogging.

Additionally, to examine the impact of premise plumbing on the microorganisms found in the dormitory GW, water samples were collected from simulated showering events without a human user (abbreviated 'BC' for building control). Showers were simulated in three different rooms plumbed to the GW collection system by turning on the shower water for a total of 8 minutes and introducing soap (Dove body soap) and shampoo (Tresemme Natural shampoo) into the water as it flowed down the drain. The soap products (~6ml each of soap and shampoo) were poured onto a glove-covered hand and then lathered and rinsed slowly for 1 minute into the shower water. The simulated shower water from each room was then collected in the 35-gallon compositing tank. For each simulated shower event, 1-L sub-samples were filtered for DNA extraction as previously described. One of the three rooms used was as a model room for which

students had not used the shower, and the other two rooms were occupied such that their showers were used regularly. The 35-gallon compositing tank used for collecting the simulated showers was rinsed with potable water and 8.25% Clorox bleach in between simulated showers. As a control, potable water (PW) was collected directly from one of the dormitory room shower heads (prior to interior shower tub contact) and used for DNA extraction (PW sample). To ensure sufficient DNA yield, DNA was extracted as previously described from 7-L of filtered potable water. DNA yield was quantified via Take3 plates and Gen5 software (BioTek Inc., Winooski, VT). DNA extracts were shipped on dry ice to the USEPA (Cincinnati, Ohio) and stored at -80°C.

High Throughput Sequencing

LA(24) and all SH (21), ET (6), BC (3), and PW (1) dormitory samples were sequenced on the 454 pyrosequencing platform (Roche, Inc.) using published primers (**Table 5**) corresponding to the V1 through V3 regions of the 16S rRNA gene. (68) The PCR reaction contained 1 µL of 10 mM dNTP mix, 1 µL DMSO, 5 µL 10X FastStart Buffer containing 1.8 mM MgCl₂, 3 µL of 10 µM forward and reverse primers, 5 units/µL FastStart HiFi enzyme, 10 ng of DNA, and PCR-grade water to a final volume of 50 µL. PCR cycling was run on the MJ 7000 cycler (Bio Rad) under the following conditions: 95°C for 2 min followed by 25 cycles of 95°C for 40 sec, 56°C for 30 sec, and 72°C for 1 min followed by a final extension of 72°C for seven minutes. No template controls (NTCs) were run with each reaction and, along with the PCR products, visualized on pre-cast E-gel® 2% agarose ethidium bromide gels (Applied Biosystems, Inc.) for amplification confirmation. Triplicate PCR was pooled by volume (20 µL per replicate) for 454 pyrosequencing. Over the course of the experiment, all NTC's (n=32) were

negative. In sum, 55 samples were sequenced for this study; however, some replicate samples were sequenced separately to evaluate classification consistency.

Table 5. Primers used for 454 Pyrosequencing

Forward	5'-CTATCCCCTGTGTGCCTTGGCAGTCTCAGAGAGTTTGATCCTGGCT-CAG-3'
Reverse	5' CCATCTCATCCCTGCGTGTCTCCGACTCAGATTACCGCGGCTG-CTGG- 3'

Roche 454 Sequence Analysis

The 454 reads were processed with Mothur (69) as follows: The reads were filtered by Phred quality (Q30 with window length of 50 nucleotides), homopolymers (maximum of 10 nucleotides), length (minimum of 300 nucleotides), and ambiguous base calls (0 allowed). The UCHIME-denovo algorithm of USEARCH software (70) was used to identify and remove chimeras. Reads were classified with the Ribosomal Database Project (RDP) Classifier using training set 9 with a minimum bootstrap of 80%. (71) Taxonomic divisions and rarefaction analysis were determined using MEGAN. (72) Principal coordinate analysis (PCoA) was performed after sub-sampling with MEGAN (72) to offset differences in sequence depth between GW groups. Network analysis using the edge-weighted spring embedded model, and network analyzer plug-in (73) were performed using Cytoscape software. (74)

Results

A summary of the top 30 classified genera in all graywater types is listed in **Figure 7** and a summary of the top 5 classified genera is presented in **Table 6**. A stacked bar graph of all classified laundry samples is depicted in **Figure 8**. Laundry water was dominated by members in the genera *Staphylococcus*, *Corynebacterium*, *Micrococcus*, *Lactobacillus*, *Acinetobacter*, *Propionibacterium*, and *Paracoccus*, accounting for approximately 50% of the total reads classified in the current study (**Figure 7**). Many of these same bacteria have previously been identified as major constituents of the human skin microbiome through the human microbiome

project (HMP). (75) Therefore, laundry graywater from the University sports facility appears to be dominated by skin-associated bacteria.

Table 6. Top five classified genera in graywater

Rank by abundance	Laundry	Shower	Equalization tank	Building control
1	<i>Corynebacterium</i>	<i>Zoogloea</i>	<i>Desulfovibrio</i>	<i>Propionibacterium</i>
2	<i>Staphylococcus</i>	<i>Acinetobacter</i>	<i>Tolomonas</i>	<i>Zoogloea</i>
3	<i>Propionibacterium</i>	<i>Propionibacterium</i>	<i>Laribacter</i>	<i>Acidovorax</i>
4	<i>Micrococcus</i>	<i>Acidovorax</i>	<i>Sulfurospirillum</i>	<i>Dechloromonas</i>
5	<i>Lactobacillus</i>	<i>Dechloromonas</i>	<i>Pseudomonas</i>	<i>Actinomyces</i>

Zoogloea, *Acinetobacter*, *Propionibacterium*, *Acidovorax*, *Dechloromonas*, *Cloacibacterium*, *Chryseobacterium*, *Pseudomonas*, *Actinomyces* and *Azospira* were the ten most abundant bacterial genera observed in SH samples (**Figure 7**). With the exception of *Acinetobacter*, these genera were also the most abundant genera present in the BC. Some of the dominant genera in the ET were similar to those in shower and BC (*Zoogloea*, *Pseudomonas*, and *Dechloromonas*), but many were unique (*Desulfovibrio*, *Tolomonas*, *Laribacter*, *Sulfurospirillum*, *Enterobacter*, *Propionivibrio*, and *Bacteroides*). The most dominant genera in the PW were *Methylobacterium* (40.1% of the reads) and *Mycobacterium* (31.4%) followed by *Sphingomonas*, *Propionibacterium*, *Zoogloea*, *Actinomyces*, *Novosphingobium*, *Dechloromonas*, *Acidovorax* and *Flavobacterium*.

A log-scale high-resolution heatmap was produced to examine the relationships between all 48 GW samples (**Figure 9**); this analysis revealed 2 groups of bacteria that distinguished laundry (Group I) from SH and ET (Group II). Group I was comprised of *Staphylococcus*, *Micrococcus*, *Paracoccus*, *Kocuria*, *Dietzia*, *Facklamia*, *Brachybacterium*, *Aerococcus*, *Brevibacterium*, *Jeotgalicoccus*, *Exiguobacterium*, *Luteimonas* and *Anaerococcus*, while Group II included *Desulfovibrio*, *Tolomonas*, *Laribacter*, *Pleomorphomonas*, *Propionivibrio*,

Dysgonomonas, *Desulfobulbus*, *Magnetospirillum*, *Sulfurospirillum*, and *Dechloromonas*. Some bacteria, such as *Propionibacterium*, *Acinetobacter*, *Pseudomonas* and *Brevundimonas*, were found in laundry, shower, ET and BC. The December 2012 shower samples contained both Group I and Group II bacteria (asterisks in **Figure 9**).

PCoA further revealed the relationships between individual GW samples (**Figure 10**). Four distinct groups were observed by the analysis: SH, SH collected in December (December-SH), ET and laundry. SH, December-SH, and laundry groups separated along principal coordinate (PC) 1 axis, which contained 54.4% of the eigenvalues. Eigenvalues covered 10.2% on PC 2, and the total coverage for these two axes was 64.6%. PC 2 distinguished ET from SH, and the eigenvalues on the third axis (5.5%) did not further separate the groups. December-SH samples formed an intermediate group between laundry and all other SH samples. BC was indistinguishable from all SH samples except December-SH (cf. purple boxes, **Figure 10**).

Figure 7: Relative abundance of classified graywater samples

Figure 9: Log-scale heat map of genera detected in graywater samples. BC: building control, PW: potable water, ET: equalization tank.

Figure 10: PCoA of GW. Symbols: equalization tank (red triangles and symbols); shower (blue); December shower (green); laundry (brown); and building drain (purple squares). Bray-Curtis was used to generate a matrix of pairwise dissimilarities from all of the classified reads at the genus level. The sequencing replicates are enclosed by ellipses: The numbers indicate the sequencing replicates performed for equalization tank GW collection dates 4-11-13 (n = 4) and 4-19-13 (n=3) (numbers 1 and 2, respectively); shower GW collection dates 4-9-13 (n=4) and 4-10-13 (n=3) (numbers 3 and 4 ,respectively); and laundry GW collection dates 2-1-13 (n=3) and 2-11-13 (n=6) (5 and 6, respectively).

Discussion

In this study, we utilized high throughput pyrosequencing to identify the bacterial composition of two distinct GW sources: laundry graywater sourced directly from an outlet pipe as well as SH and BC collected from building drain pipes and an ET. The bacterial composition

of laundry water was dominated by genera (*Corynebacterium*, *Staphylococcus*, *Micrococcus*, *Propionibacterium*, *Kocuria*, and *Paracoccus*, **Figure 7**) identified as common colonizers of human skin by the human microbiome project. (76-78) Genera associated with the vagina and yogurt (*Lactobacillus*) (75, 79) and penis (*Facklamia*) (80) were also present. Many of the other prevalent but less abundant genera in laundry, such as *Dietzia*, *Brevibacterium*, *Exiguobacterium*, *Brachybacterium*, *Aerococcus*, *Jeotgalicoccus*, and *Brevundimonas* exhibit halotolerant/philic properties or are skin-associated, (81-88) consistent with laundering of athletic clothes.

One sample set (pre-rinse and wash) occurred with an anomalously large number of reads assigned to the genus *Vibrio*, which could have been associated with an infection (**Figure 8**). The reads were examined further to attempt to determine which species was responsible for the event, but due to the heterogeneity of this location of the *Vibrio* genome, (89) species-level taxonomy was unsuccessful. In general, various *Vibrio* spp. exist naturally in marine and estuarine waters, (90) and while many may be non-pathogenic, seven including *V. parahaemolyticus* and *V. cholerae*, are human enteric pathogens. (91) Additionally, *V. alginolyticus* and *V. vulnificus* have the ability to infect skin abrasions, with *V. vulnificus* causing wound infections and septicemia, accompanied by edematous skin damage. (91, 92) While *Vibrio* seemed a somewhat unexpected genus, the particular laundry graywater sample was derived from the return of several athletic teams from coastal destinations. Given the potentially high 16S rRNA gene copy number per genome of some species, (89) we hypothesize that the *Vibrio* contribution could be explained by cells transferred to laundry graywater via fecal, oral, or dermal desquamations deposited in clothing from an infected individual, and/or by an athlete's clothing.

Previous skin desquamation investigations have found that short-term vigorous scrubbing and long-term natural shedding rates are similar. (93) Given this information, one might predict a similar skin-associated signature between SH (short-term desquamation) and laundry samples (long-term desquamation). Although SH samples collected from drain pipes had some overlap with the common skin-associated organisms observed in laundry (e.g., *Propionibacterium*, *Acinetobacter*, *Corynebacterium*, *Staphylococcus*), SH also contained distinct genera prevalent in wastewater infrastructure such as *Acinetobacter*, *Aeromonas*, *Cloacibacterium*, *Tolomonas*, *Enhydrobacter*, *Pseudomonas*, *Streptococcus*, *Propionivibrio*, *Bacteroides*, (94) and *Dechloromonas* and *Zoogloea*. (95) The December-SH samples were more similar to laundry than SH samples taken at all other times (**Figures 9 and 10**) due to the higher prevalence of skin-associated organisms. This intriguing phenomenon is likely explained by the increased number and mass of stratum corneum layers in human skin (96) and higher desquamation rates (97) associated with low humidity, dew points, and temperature. Since these environmental parameters are at their lowest levels in Colorado during the month of December, (98) one would expect the highest density of desquamated skin cells (and associated bacteria) in SH samples from the December-SH sampling, as our data indicates.

PCoA is a multidimensional scaling tool that is used in statistics to weight variables in order to examine spatial relationships in ecological modeling. (99) PCoA of graywater revealed that each graywater type clustered more closely to itself than it did to other graywater types, (**Figure 10**) suggesting that the microbial presence is likely to differ in each graywater source. The differences in these sources is echoed in the log-scale heatmap (**Figure 9**) where the different genera typical of human skin, drain infrastructure, and stable tank environments highlight the differences between graywater sources. These analyses indicate that the microbial

population of various graywater matrices is likely to be highly influenced by the type of graywater being contributed into the system and the temporal variation observed within the system. This could potentially result in a wide variety of low quantities of potential bacterial contaminants entering a graywater system, since contributors (clothes, skin, produce, etc.) may be exposed to varying environmental conditions containing differing microbes such as from soil, air or water. However, regardless of the exposure of contributors to these medium, the human-associated input to graywater is likely to remain stable from human contact of the source water, making these targets good candidates for future examination of satisfying treatment needs.

The similarity in composition between the BC and SH samples suggests that flushing of loose cells or sloughing of biofilms within the collection system strongly influence the composition of collected SH water, even with as little as 1.5m of collection pipes. The bacteria present in the BC samples contained many organisms reported from a recent study of hospital sink drain biofilms, (100) including *Acinetobacter*, *Brevundimonas*, *Corynebacterium*, *Cupriavidus*, *Dechloromonas*, *Ohtaekwangia*, *Pseudomonas*, *Pseudoxanthomonas*, *Sphingobacterium*, *Sphingobium*, *Sphingomonas*, *Sphingopyxis* and *Streptococcus*. The importance of building infrastructure bacteria found in the work reported herein is also in alignment with recent research, which found that 81.4% of bacterial sequences in untreated wastewater were related to sewage infrastructure bacteria (i.e. non-fecal). (101)

The majority of classified reads in ET samples were from the genera *Desulfovibrio*, *Tolomonas*, *Laribacter*, *Sulfurospirillum*, *Pseudomonas*, *Enterobacter*, *Dechloromonas*, *Propionivibrio*, *Zoogloea*, *Bacteroides*, *Dysgonomonas*, *Sporomusa* and *Desulfobulbus*. Species within some of these genera are generally classified as facultatively anaerobic with reduction properties associated with nitrogen, (102) phosphorus, (102) chlorate, (103) perchlorate, (104)

and sulfur; (105) many have also been isolated from wastewater treatment plants or water infrastructure. (95) These results are consistent with the active degradation of surfactants and other organic matter within the ET and observed low dissolved O₂. ET samples exhibited a consistent composition over the period of one year, suggesting that a stable assemblage of bacteria populate this habitat.

Results indicate that 1) skin-associated organisms are the most abundant human-associated bacteria, 2) sampling location within a GW collection system can influence bacterial types 3) bacteria from the infrastructure (both drainage lines and equalization tanks) are the most abundant in water collected for reuse, and 3) several relatively rare genera, most likely associated with drinking water infrastructure, are present in all samples. Additionally, bacterial targets of water infrastructure nature may be the most appropriate surrogates to use for measuring a wide dynamic range of treatment log reduction due to prevalence and abundance. Therefore these genera should be targets of further research to determine if these genera can act as surrogates for assessing pathogen risk and removal during treatment in water reuse systems.

Chapter 3: Molecular Quantification of Select Surrogate and Pathogenic Targets in Laundry Graywater

Portions of Chapter 3 are expected to be submitted to *Environmental Science & Technology*

Introduction

Due to the fecal-oral route of pathogen transmission in graywater, previous studies aimed at identifying microbial risk by targeting enteric pathogens or FIB that are presumed to be indicative of fecal contamination and possible pathogen presence. However, fecal indicators are unlikely to vary quantitatively with pathogens because fecal indicators are constantly shed and pathogens are much more sporadic in their occurrence, (3) and FIB are also unlikely to accurately predict the risk from potentially low levels of pathogens shed via other pathways such as respiratory/oral and dermal. (17) Therefore new approaches of assessing microbial risk in graywater are necessary for future adoption of water reuse strategies.

A surrogate is a easily measurable model or index organism that is expected to represent a pathogen or group of pathogens. (31, 106) Metcalf & Eddy (107) suggests the following criteria for an indicator organism:

1. the indicator organism must be present when the pathogen is present
2. the numbers of indicators present should be equal to or greater than the pathogenic target
3. the indicator must exhibit similar or greater survival characteristics in the environment as the target pathogen or group of pathogens for which it is representing
4. the indicator must not reproduce outside of the host organism
5. the isolation and quantification of the indicator must be as fast as or ideally faster than the target pathogen(s); it should also be cheaper and easier than the pathogen assay.

Although these guidelines were designed for fecal pollution, a majority of the same principles can act as guidelines for surrogates to represent pathogenic groups of fecal or non-fecal origin

during treatment or behavioral persistence studies. For these reasons, spores of non-pathogenic *Bacillus* have previously been used as surrogates for anthrax (31) and *Clostridium perfringens* as surrogates for protozoan oocysts and enterovirus. (33) Bacteriophages of *E. coli* (coliphages – F-specific and somatic) (33) and *Bacteroides*, (34) (GB-124), (35) have also been used or proposed as surrogates for viral research. In this context for using a surrogate as an indication of treatment of efficiency via pathogen removal, the surrogate does not necessarily have to be shed by the same pathway as the pathogen(s) of interest. The surrogate must still be present in equal-to or greater quantities than the pathogen(s) of interest, but the most important attribute is that the surrogate must demonstrate a relationship to a pathogen(s) of interest in response to a particular variable such as treatment or environmental fate behavior.

Since public acceptance of reusing treated water within a household is very tentative, (108) it is necessary to take a cautious and conservative approach to removing any risk associated with graywater reuse. Therefore, a scientifically accepted public risk of reuse could be to obtain an annual infection rate of 1 per in every 10,000 persons, which could translate to a 4-log removal of pathogens which is the currently practiced and accepted removal of virus and protozoan pathogens in drinking water treatment in the U.S. today. (30, 109) Therefore, it is necessary to find a treatment indicator that is present in graywater in high enough numbers to measure a removal of this size. Since water reuse requires a decentralized approach, external spiking or seeding of any surrogate into the matrix would likely be uneconomical. Thus, it is essential to find a suitable treatment indicator endogenous to the matrix itself that is consistently present in high enough numbers to ensure adequate sensitivity required to measure this removal. Therefore, this research targets several potential endogenous targets and uses abundance,

consistency, and persistence to evaluate their use as a treatment surrogate for assessing risk of pathogen breakthrough during treatment.

Methods and Materials

Laundry Sampling

Samples were collected and processed as described in Chapter 2, **Laundry Sampling**. Briefly, the University of Cincinnati in Cincinnati, Ohio, United States, uses two washing machines (Pellier Milnor Corporation models 36026 V5J and 30022 V6J) to wash the University's 500+ student athletes' sportswear and towels. One washing machine is dedicated mostly to clothing attire while a smaller washer services towels. The laundry facility has a collection bin in which all athletes place their laundry onto a loop to be washed by the equipment room staff. The large clothing washer has a 0.43m^3 capacity and discharges roughly 250 liters per cycle. The washer is run daily using the following conditions: a 3-minute rinse (21°C), 6-minute wash (29°C), 12-minute wash (29°C), followed by two 2-minute rinse cycles at (27°C) and (21°C) discharging approximately 250 liters of water per cycle, and a final spin-only extract. The same concentration of detergent (Tide 2X concentrate, Procter & Gamble Co.) is automatically injected for each wash cycle. All discharges are pooled into a collection basin which connects to the sewer drain. One-liter grab samples were taken from both the pre-rinse ($n = 20$, no detergent) and first wash ($n = 20$, detergent included) in sterile 1-L Nalgene bottles halfway through the approximate 40-second discharge period, prior to sewer drain entrance, from February to May, 2013. Collection of the first two cycles was based on the expectation that a majority of microbes from clothing would be removed during these first two cycle discharges, (16) thereby representing the highest predicted biological presence and highest microbial risk

associated with graywater reuse. Samples were placed on ice and immediately transported to the lab for concentration and nucleic acid extraction within two hours.

Sample Concentration and DNA Extraction

Samples were vacuum filtered through 47 mm diameter, 0.45 µm mixed cellulose ester (nitrocellulose) membranes (Pall Corporation, Inc.). Filtrate volume ranged from 70-380 mL before filters clogged, ensuring adequate DNA yields. Filter blanks using 100 mL of double-distilled water were used to monitor for extraneous DNA contamination. Filters were removed from the filter cone apparatus using sterile forceps and placed into 47 mm Petri dishes (Pall Corp., Inc.) and processed immediately or stored at -70°C until DNA extraction. DNA was extracted using the PowerWater DNA Extraction Kit (MoBio Labs, Inc.) according to the manufacturer's instructions. DNA extracts were quantified using a ND-1000 nanodrop spectrophotometer (NanoDrop Technology, Wilmington, DE) and stored at -70°C. **Table 4** shows the volume of laundry water filtered and the concentration of extracted DNA in all laundry samples.

qPCR Standards

Bacterial species used for standards included *Bacteroides fragilis* (HE608156), (35, 110) *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (*S. aureus*) (ATCC 25923), *Escherichia coli* (NCTC 12923), and *Enterococcus faecalis* (NCTC 12697). Genomic DNA was provided by EPA and extracted using the DNEasy Blood and Tissue Kit (Qiagen, Inc.) according to the manufacturer's instructions, and quantified via Nanodrop 1000 (NanoDrop Technology). Bacterial gene copies were estimated based on DNA yield and the approximated (or known) genome mass and GC-content displayed in **Table 5**. The HmtDNA standard curve method was obtained via double amplification of a purified amplicon as described previously, (61) but

differing in that the HmtDNA amplicon came from a cheek swab of a human volunteer as opposed to an environmental sample. Amplicons were purified using the MinElute PCR Purification Kit (Qiagen, Inc.), and DNA yield was assessed via Nanodrop 1000 spectrophotometry (NanoDrop Technology). HmtDNA gene copies were calculated based off DNA yield, base pair content and amplicon size. (61) Standard curves for targets were generated using 10-fold serial dilutions in PCR-grade water ranging from 10^1 to 10^6 gene copies per reaction. Triplicate qPCR standards for each assay were used to create a master standard curve for each assay. Amplification efficiency (E) was calculated using the equation $E = (10^{-\text{slope}/1}) - 1$. Data analysis was completed in R version 2.15.2 (111) or Microsoft Excel 2011 using evaluation criteria of goodness-of-fit linear regression (R^2) and slope to evaluate each assay. Samples below the lower limit of quantification (LLOQ), which was defined as the mean C_q of the lowest detectable gene copy number from the calibration curve qPCR reactions, were assigned a value of $\frac{1}{2}$ the LLOQ for subsequent data analysis.

Table 7. Culture Genome Sizes used for qPCR Standards

Bacteria	Genome size (Mb)	GC Content
<i>Bacteroides fragilis</i>	5.36*	43.40%*
<i>Pseudomonas aeruginosa</i>	6.46	66.15%
<i>Escherichia coli</i>	4.75	50.90%
<i>Enterococcus faecalis</i>	3.03	37.20%
<i>Staphylococcus aureus</i>	2.77	32.80%

*Mean of *B. fragilis* strains in NCBI (n = 14, SD = 0.11)

qPCR Assay

Most qPCR primers and probes used for this study have previously established sensitivity and target specificity (**Table 6**). (57, 75, 112-117) The one exception to this specificity was the *S. aureus* assay initially reported by Lee *et al.* 2006, (118) which targeted the *sec* gene. Using reported parameters, as well as a variety of other PCR conditions, we were unable to obtain

amplification from a pure *S. aureus* (ATCC 25923) culture DNA extract using the primer sequences specified. As a result, another Taqman assay targeting the *nuc* gene of *S. aureus* was successfully adopted and used. (117)

Duplicate qPCR was carried out in 30 μ L reactions consisting of 15 μ L Taq Environmental Master Mix 2.0 (Life Technologies, Inc), 500 nM of each primer, 100 nM of each probe, 2 μ L of DNA template and water to volume. All primers and probes were synthesized by Life Technologies, Inc. Cycling was run on ABI Prism 7000 (Applied Biosystems, Inc.) at 95°C for 10min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min.

Adenovirus PCR

PCR was carried out for Adenovirus in 30 μ L reactions consisting of 15 μ L Taq Environmental Master Mix 2.0 (Applied Biosystems, Inc.), 200 nM of each primer, (114) 2 μ L DNA template and PCR-grade water to volume. Cycling was performed on GeneAmp PCR System 9700 (Applied Biosystems, Inc.) at 95° for 10 min, followed by 35 cycles of 95° for 15 sec and 60°C for 1 min. Presence/absence was confirmed by gel electrophoresis on pre-cast 2% agarose E-gels® (Applied Biosystems, Inc.).

qPCR Quality Control and Data Analysis

qPCR setup and amplification occurred in separate designated labs to avoid contamination. No template controls (NTC's) were run with all samples. DNA template dilutions of 1:10 and 1:25 were employed to check for PCR inhibitors. PCR inhibition criterion was defined as the expected environmental sample $\Delta C_q \pm$ the standard deviation of the assay (119) for the expected $C_q + 0.5$ cycles for PCR variation recommended previously (120) resulting in an approximate 1 cycle variation from the expected ΔC_q . For inhibited samples,

original copy numbers were back-calculated based on the log-linear relationship observed between subsequent 1:10 and 1:25 dilutions. The *Enterococcus* spp. assay gene copy number

Table 8. qPCR Primers and probes used in this study

Target	Seq 5' > 3'	Gene
<i>E. coli</i> (113)	EC784F: GTGTGATATCTACCCGCTTCGC	uidA
	EC866R: AGAACGGTTTGTGGTTAATCAGGA	
	EC807P: 6F-TCGGCATCCGGTCAGTGGCAGT-TA	
<i>Pseudomonas</i> spp. (121)	Pse435F: ACTTTAAGTTGGGAGGAAGGG	16s rRNA V3-V4
	Pse686R: ACACAGGAAATTCCACCACCC	
	Pse491P: 6F-ACAGAATAAGCACCGGCTAAC-TA	
<i>Bacteroides</i> spp. (115)	BAC296F: GAGAGGAAGGTCCCCAC	16s rRNA
	BAC412R: CGCTACTTGGCTGGTTTCAG	
	BAC375P: 6F-CCATTGACCAATATTCCTCACTGCTGCCT-TA	
<i>Bacteroides</i> (Human-specific) (115)	HBac566F: GGGTTTAAAGGGAGCGTAGG	16s rRNA
	HBac692R: CTACACCACGAATTCGCCT	
	HBac594P: 6F-TAAGTCAGTTGTGAAAGTTTGC GGCTC-TA	
<i>Enterococcus</i> spp. (113)	ENC784F: AGAAATTCCAAACGAACCTG	23s rRNA
	ENC854R: CAGTGCTCTACCTCCATCATT	
	ENC813P: 6F-TGGTTCTCTCCGAAATAGCTTTAGGGCTA-TA	
<i>Staphylococcus aureus</i> (117)	S.A.F: GCGTACTAGTTGCTTAGTGTTAACTTTAGTTG	nuc
	S.A.R: TGCATATATACTGTTGGATCTTCAGAA	
	S.A.P: 6F-TGCATCACAAACAGATAACGGCGTAAATAGAAG-TA	
HmtDNA (57)	HmtF: CAGCAGCCATTCAAGCAATGC	NADH5
	HmtR: GGTGGAGACCTAATTGGGCTGATTAG	
	HmtP: 6F-TATCGGCGATATCGGTTTCATCTCG - TA	
Adenovirus (114)	AdV-JTVXF: GGACGCCTCGGAGTACCTGAG	hexon
	AdV-JTVXR: ACGGTGGGGTTTCTGAACTTGTT	
	AdV-JTVXP: 6F-CTGGTGCAGTTCGCCCGTGCCA - TA	
<i>Staphylococcus</i> spp. (75)	16s519f: CAGCAGCCGCGGTRATA	16s rRNA
	16s785r: GGACTACCVGGGTATCTAAKCC	
	Staph.P: 6F-CTGTAAGTACGCTGATGTG - MGB	
<i>Corynebacterium</i> (75)	16s519f: CAGCAGCCGCGGTRATA	16s rRNA
	16s785r: GGACTACCVGGGTATCTAAKCC	
	Coryne.P: 6F-ACAGYACTCHAGTHATGCCCGT - MGB	
<i>Propionibacterium</i> (75)	16s519f: CAGCAGCCGCGGTRATA	16s rRNA
	16s785r: GGACTACCVGGGTATCTAAKCC	
	Propi.P: 6F-CTTTCGATACGGGTTGACTT - MGB	

TA – Tetramethyl rhodamine (TAMRA)

6F – 6-Carboxyfluorescein

MGB – Minor groove binding

was divided by four to account for the fact that *Enterococcus* spp. contain approximately four 23S rRNA gene copies per genome. (122) Results were normalized to 10ng of DNA per PCR reaction per 100mL of graywater. Pearson's product-moment correlation test (r) was used to

assess the validity of statistical relationships between targets. Statistical strength was determined at $\alpha = 0.01$, $1-\beta = 0.8$. (123) All results are reported as $\log_{10}$ gene copies $\pm$ the standard deviation (SD) per 100mL of graywater.

HmtDNA Sanger Sequencing

To confirm the specificity of the HmtDNA primer to human origin, all qPCR HmtDNA amplicons (n=40) were purified with the MinElute PCR Purification Kit (Qiagen, Inc.) according to the manufacturer's instructions and Sanger sequenced with both HmtDNA forward and reverse primers. (57) NCBI BLAST searches of the Sanger sequences (n=80) were performed to verify sequence identity.

Results

qPCR Standards

The standard curves for *Corynebacterium*, *Propionibacterium*, and *Staphylococcus* spp. Assays exhibited linear relationships from 10^8 to 10^3 gene copies per reaction, with linearity for the remaining targets from 10^6 to 10^1 gene copies per reaction. PCR efficiencies ranged from 96.8% to 109%, slopes ranged from -3.13 to -3.40 with all assays exhibiting correlation coefficients (R^2) ≥ 0.99 , except for the *S. aureus* assay which exhibited a $R^2 = 0.98$. Calibration equations for the standard curves can be found in **Table 7**. An example of replicate HmtDNA standard curves, from which a master curve was made, are depicted in **Figure 11**. The results of these standard curves are as expected. During PCR amplification, an ideal slope is -3.32, that is every cycle that the PCR reaction goes through, the product will double until limitations exist with reaction components. Since these slopes are all very close to the accepted slope range ($85 \leq \text{slope} \leq 115\%$), these slopes can be used for quantification and confirms that all assays are performing as expected.

Table 9. qPCR standard curve equations averaged from replicates of triplicate copy numbers per curve

Target	Master Curve Equation	Number replicates
HmtDNA	$y = -3.32x + 41.17$ $R^2 = 0.99$	4
<i>E. coli</i>	$y = -3.33x + 39.57$ $R^2 = 0.99$	4
<i>Bacteroides</i>	$y = -3.32x + 39.95$ $R^2 = 0.99$	4
Human-specific <i>Bacteroides</i>	$y = -3.34x + 41.59$ $R^2 = 0.99$	4
<i>Enterococcus</i> spp.	$y = -3.40x + 40.43$ $R^2 = 0.99$	4
<i>Pseudomonas</i> spp.	$y = -3.38x + 41.98$ $R^2 = 0.99$	4
<i>S. aureus</i>	$y = -3.19x + 42.63$ $R^2 = 0.98$	3
<i>Staphylococcus</i> spp.	$y = -3.25x + 45.60$ $R^2 = 0.99$	2
<i>Propionibacterium</i>	$y = -3.38x + 47.79$ $R^2 = 0.99$	2
<i>Corynebacterium</i>	$y = -3.13x + 47.15$ $R^2 = 0.99$	2

Figure 11. Standard curves and equations of HmtDNA amplicons used for quantification (n=4)

PCR Inhibition

The HmtDNA, universal *Bacteroides*, human-specific *Bacteroides*, and *Pseudomonas* spp. assays showed inhibition in 10% (4/40) of samples, *E. coli* had 5% (2/40) inhibited samples, 2.5% (1/40) of the *Propionibacterium* and *Corynebacterium* assays and no inhibition was observed for the *Enterococcus* and *S. aureus* assays. The *Staphylococcus* assay showed inhibition in 88% (35/40) of samples. Some samples showed inhibition for multiple targets

while other samples were only inhibitory for a particular target. To compensate for the variable inhibition, qPCR reactions were optimized by dilution. Expected ΔC_q values were observed between 1:10 and 1:25 dilutions of inhibited samples, suggesting that the samples were potentially free of PCR inhibitors after the 1:10 DNA template dilution. Original gene copy numbers were back-calculated using the ΔC_q of the linear relationship between the non-inhibitory 1:10 and 1:25 dilutions.

Figure 12 depicts an example of inhibition optimized by dilution. Based on perfect doubling of the product during each round of a PCR cycle, approximately a -3.3 change in C_q or the slope of the standard curve of the assay (-3.12 for total *Staphylococcus*) would be experienced when no inhibition was observed. When inhibited, such as in the blue triangles of the *Staphylococcus* assay below for undiluted and 1:10 dilution, there is less than a 2 C_q change (120) between the samples. The 1:10 dilution C_q would be expected at approximately 31.2 (a 3.12 C_q increase) if no inhibition was experienced (purple X's). Thus, another dilution (1:25 in this case) was performed. When expected ΔC_q values were observed between 1:10 and 1:25 dilutions as seen by the blue triangle at dilution 0.04 (**Figure 12**), the original template C_q was adjusted based on the slope of the standard curve for that particular assay (green triangle **Figure 12**) so that comparisons could be made between samples based on the amount of total DNA added per PCR reaction.

Figure 12. Example of inhibition resolved by dilution. No inhibition (Tstaph purple X's) and inhibition resolved by dilution (Tstaph, blue diamonds [inhibited], green dotted line and triangle [adjusted]).

PCR Results for Surrogate and Pathogenic Targets

E. coli, *Enterococcus* spp., and *S. aureus* were detected in 10 (50%), 19 (95%), and 17 (85%) of pre-rinse samples and 8 (40%), 20 (100%), and 15 (75%) respectively of first wash samples (**Table 8**). Adenovirus was not present in any sample. All other targets tested for in this study were detected in every sample. *Staphylococcus* spp., *Propionibacterium*, and *Corynebacterium* ranked highest in abundance of qPCR targets (mean = 6.54 ± 0.54 , 5.73 ± 0.68 , 5.44 ± 0.78 respectively) $\log_{10}$ copies per 100 mL of graywater while HmtDNA was present (mean = 2.75 ± 0.52) in all samples (**Figure 13**). Human-specific *Bacteroides* comprised roughly 80% of the total *Bacteroides* spp. present. When present, an opportunistic pathogen, *S. aureus* was detected (1.67 ± 0.82) $\log_{10}$ copies, comprising approximately 25% of total *Staphylococcus* spp.

Table 10. qPCR Detections in Graywater

	Hmt	E. coli	Bac	hBac	Enc	Pse	Cory	Propi	Staph	StaphA	Adv
Rinse	100%	50%	100%	100%	95%	100%	100%	100%	100%	85%	0%
Wash	100%	40%	100%	100%	100%	100%	100%	100%	100%	75%	0%

Data is % detection of samples tested. N = 20 for the rinse and n = 20 for the wash in duplicate

Figure 13. Mean log10 copies $\pm$ SD of qPCR targets (Hmt = HmtDNA, Bac = total *Bacteroides* spp., hBac = human-specific *Bacteroides*, Enc = *Enterococcus* spp., Pse = *Pseudomonas* spp., Cory = *Corynebacterium*, Propi = *Propionibacterium*, Staph = *Staphylococcus* spp., StaphA = *Staphylococcus aureus*) in laundry graywater.

Although the focus of this study was to identify consistently abundant markers in laundry graywater, several significant correlations were found between qPCR targets. A summary of all statistical correlations and significance between targets is available in **Table 9** with dark shading denoting significant correlations at $\alpha \leq 0.01$. Of note, significant positive correlations were observed between the presence of HmtDNA and skin-associated organisms *Staphylococcus*,

Propionibacterium, and *Corynebacterium* ($r \geq 0.45$, $P \leq 1.4 \times 10^{-3}$) and the skin pathogen *S. aureus* ($r = 0.54$, $P = 3.2 \times 10^{-4}$) (**Figure 14A**). HmtDNA tracked fairly well in response to *S. aureus*, remaining approximately 1-2 log higher throughout the course of the study (**Figure 14B**). Interestingly, none of the FIB assayed in this study showed any significant correlation with each other or to HmtDNA ($P \geq 0.01$) (**Table 9**).

Table 11. Summary of qPCR correlations in laundry graywater

Target	StaphA	Pse	Coryne	Propi	Staph	Enc	Bac	hBac	<i>E. coli</i>
Pse	0.67	-	-	-	-	-	-	-	-
Cory	0.37	0.20	-	-	-	-	-	-	-
Propi	0.55	0.34	0.60	-	-	-	-	-	-
Staph	0.62	0.66	0.38	0.47	-	-	-	-	-
Enc	0.45	0.63	0.24	0.29	0.60	-	-	-	-
Bac	0.28	0.31	0.39	0.46	0.35	0.31	-	-	-
hBac	0.36	0.35	0.38	0.49	0.45	0.30	0.60	-	-
<i>E. coli</i>	0.50	0.50	0.02	0.21	0.40	0.35	0.01	-0.04	-
HmtDNA	0.54	0.32	0.48	0.49	0.45	0.13	0.20	0.26	0.25

Gray highlighting denotes significant correlation at $\alpha \leq 0.01$, $1-\beta = 0.8$

Figure 14. A) qPCR correlation between HmtDNA vs. *S. aureus* ($r = 0.54$, $P = 3.2 \times 10^{-4}$) ($n=40$) and B) qPCR fluctuations between select HmtDNA and *S. aureus* laundry wash samples in 2013 ($n=14$).

HmtDNA Sanger Sequencing

Table 12 (Appendix A) displays the Sanger sequences of the HmtDNA amplicons that were sequenced using both forward and reverse primers. All sequences (n=80) exhibited $\geq 99\%$ match identities to *Homo sapiens* mitochondrion complete genome (accession# KC994161.1, e-values $\leq 1.0 \times 10^{-60}$) except one, which exhibited a 97% match identity. Results confirm no cross-reactivity to non-human mitochondria.

Discussion

Several qPCR targets were assayed in this investigation to identify microbes present in laundry graywater that could serve as candidate treatment surrogates based on abundance and consistency. The qPCR analysis revealed that low levels of the FIB *E. coli*, human-specific *Bacteroides*, and fecal enterococci were present in laundry graywater, which is congruent with previous investigations. (1, 2, 17) However, traditional FIB were 2- to 5-logs lower in abundance and showed less consistency when compared to putative skin microbiome members (**Figure 13**), suggesting that their use as a surrogate to demonstrate log removal during treatment is inadequate. In addition, no significant correlations could be drawn between any FIB and the opportunistic pathogen *S. aureus* (**Table 9** $P \geq 0.01$), supporting previous conclusions that the use of FIB as an indicator of pathogen presence in laundry graywater is also limited. In contrast, HmtDNA was consistently present in laundry graywater ($2.8 \pm 0.5 \log_{10}$ copies per 100mL) and was significantly positively correlated with the opportunistic pathogen *S. aureus* ($r = 0.54$, $P = 3.2 \times 10^{-4}$, **Figure 14A, B**), suggesting that it could be a marker of human pathogen presence and could be a useful surrogate to measure pathogen removal/inactivation during treatment. HmtDNA also exhibited a significant correlation with all skin-associated bacteria ($P \leq 0.01$) but not with any FIB ($P \geq 0.01$), suggesting that the majority of the HmtDNA contribution in laundry

graywater is likely of non-fecal origin. However, if a significant increase in fecal load were to be observed for a sample, it would be expected that HmtDNA would also increase since it is shed in the feces of humans. (61)

The presence of *S. aureus* in laundry graywater is somewhat contradictory to previous investigations that were unable to isolate *S. aureus* from graywaters (18) but in alignment with other research that has identified *S. aureus* from graywaters. (4, 64) The absence of *S. aureus* in the first study could be caused by the methodology used for detection (culture vs. qPCR) or that samples in the previous study were taken from a holding tank, and we have shown that *S. aureus* does not appear to persist for long periods of time in laundry graywater at 20°C (**Figure 22**). Also, it is not surprising that *S. aureus* was found in laundry graywater, given that *S. aureus* has been documented on the human skin or in the nasal passage of approximately 30-36% of healthy individuals in the United States, (124, 125) and graywater is expected to contain organisms from body orifices and nasal passages. (4, 126)

The presence of *S. aureus* is of potential concern due to its ability to infect oral, dermal, or respiratory tracts of individuals with weakened immune systems, children, or the elderly and its antibiotic-resistant form, Methicillin-resistant *S. aureus* (MRSA). (127) MRSA is known to cause severe skin infections among contact sport athletes, (128, 129) and has more recently infected otherwise healthy people having no association to hospitals or sporting facilities, which are perceived typical MRSA infection hotspots. (127) While MRSA was not specifically tested for in this study, the potential for an infected individual to contribute MRSA to laundry graywater seems plausible based on the prevalent skin contribution (**Figure 13**) and salinity (**Table 14**) of laundry graywater, as well as MRSA's persistence in halophilic waters. (130, 131)

Even without the Methicillin-resistant form, skin-associated *S. aureus* can still colonize and infect the human skin (132) and has the ability to produce staphylococcal enterotoxins (SE) that have been responsible for an estimated 185,000 cases of foodborne gastroenteritis per year in the U.S. (133) Human-associated *S. aureus* has been shown to harbor SE genes gene(s) encoding for at least one of the 20+ currently identified enterotoxins. (134, 135) The SEs produced are more resistant to environmental stresses than the bacteria itself, (136) making the toxins themselves an additional treatment concern. In addition, a recent U.S. Department of Defense study determined that a specific SE, identified as SEB, remained stable in deionized water (pH 7.0, 10mM PO₄) for at least 30 days, which was the duration of the study. (137, 138) Therefore, water reuse scenarios in which there is oral ingestion, respiratory inhalation, or dermal contact of untreated laundry graywater may produce an increased human health risk. The myriad of reuse scenarios and inevitable downstream human contact makes it difficult to quantify the risks attributable to skin pathogens during reuse. (126) Therefore, future research is necessary to determine the importance of skin pathogens and associated enterotoxins in graywater and assess their significance for measuring pathogen removal and risk during treatment and reuse.

Chapter 4: Storage Effects on Laundry Graywater

Portions of Chapter 3 are expected to be submitted to *Environmental Science & Technology*

Introduction

Since any on-site treatment and re-use of graywater will likely include some type of storage of untreated graywater to equalize flow to the treatment process, it is necessary to determine the stability of the surrogate in these storage situations and understand any micro-ecological interactions that may influence its behavior. Previous studies have shown that total and fecal coliforms, among others, can exhibit growth in graywater, (6) suggesting that microbial interactions are taking place fairly quickly during storage. (2) In order to assess the stability of selected surrogates and pathogen in graywater, a study was conducted on storing untreated laundry graywater at various temperatures and measuring the decay or growth of select surrogates and pathogens detectable by qPCR. A preliminary study was conducted first for feasibility (“Pilot Study”) followed by a more comprehensive study, referred to as “Storage Study”.

Pilot Study

A pilot study was conducted first to evaluate the stability of HmtDNA in laundry graywater. In addition, during this pilot study, we applied pyrosequencing of a number of days during storage to understand any potential microbial interactions that might take place during this period.

Storage Study

Based on the data compiled during the pilot study, it was deemed necessary to understand the chemical affects that might take place and influence microbial effects on stored laundry graywater. To this extent, an auto-logging multi-sensor probe was used in this experiment to evaluate a number of parameters that are likely indicative of microbial activity such as dissolved oxygen (DO) and pH levels. As the stored laundry graywater ages, some microbes in the

solution are expected to grow exponentially under ideal conditions (perfect temperature, non-limiting substrate and competition). However, without readily stable oxygen input to the system, it would be expected that as the graywater ages, the solution will become more acidic due to the release of CO₂ into the solution via cellular respiration. (133) Since some organisms can only survive within limited conditions, factors such as pH and DO can assist in determining the conditions capable of supporting microbial life which are likely present within the matrix (i.e. anaerobes, aerobes). Therefore, this experiment was undertaken to assess how the storage of untreated laundry graywater affects the microbial composition.

Methods and Materials

Pilot Study Design

An initial study was conducted to assess the decay of qPCR-detectable HmtDNA in stored and untreated laundry graywater. Replicate 1-L samples of a laundry wash sample were taken in sterile 1-L Nalgene bottles as previously described from the University of Cincinnati athletic facility. An initial 100-mL sub-sample was taken immediately upon return to the lab and filtered through 0.45µm nitrocellulose filter (Pall Corp.) as described previously. The remaining liquid (approx. 900mL) was held in the Nalgene bottle at both 20°C and 4°C for up to 10 days. 100mL sub-samples were consequently filtered as described previously and DNA extractions (MoBio kit) were performed were on days 0, 1, 3, 6, 8, and 10. Filters were frozen at -80°C prior to DNA extractions. qPCR quantified the HmtDNA degradation at for all time points in triplicate and pyrosequencing identified the bacterial interactions on days 0, 1, 3, 6, and 8. **Table 10** displays the DNA yields and purity obtained during the pilot study. The qPCR was run using the same primers and conditions as previously described (Chapter 3, **Methods & Materials**).

Table 12. DNA yields and concentration of pilot study

Sample	Day – Storage	DNA Yield (ng/ μ L)	260/280
5.28p3	0	5	1.49
5.28w3 4c		4.7	1.61
5.28p3 4c	1	7.25	1.72
5.28w3 4c		14.25	1.85
5.28p3 20c		68.6	1.9
5.28w3 20c		15.65	1.77
5.28p3 4c	3	48.25	1.83
5.28w3 4c		14.15	1.81
5.28p3 20c		63.2	1.93
5.28w3 20c		65	1.95
5.28p3 4c	6	51.4	1.93
5.28w3 4c		86	2.02
5.28p3 20c		58.5	1.98
5.28w3 20c		79.5	1.93
5.28p3 4c	8	35.8	1.88
5.28w3 4c		59.6	1.91
5.28p3 20c		62	1.97
5.28w3 20c		81	2.00
5.28p3 4c	10	74.9	1.98
5.28w3 4c		69.4	1.94
5.28p3 20c		70.1	1.96
5.28w3 20c		49	1.91

Storage Study Design

Based on pilot study results, an experiment was conducted to assess the decay of qPCR-detectable surrogates (HmtDNA and *Staphylococcus*) and pathogen (*S. aureus*) in stored laundry graywater. Pre-rinse and first-wash samples (3-L each) were sampled in 1-L Nalgene bottles as described previously, then were combined (1.5-L each) into a 3-L glass bioreactor (Bellco Glass, Inc.) and stored in the dark at 4°C and 20°C (**Figure 15**). A t-test between surrogates in rinse and wash samples revealed no differences in means ($P \geq 0.05$) allowing for the combination of these effluents. A stir bar was automatically programmed to gently mix the solution for one minute

every two hours, with a 556 multi-parameter probe (YSI, Inc.) measuring DO, pH, specific conductivity, oxygen reduction potential, and salinity every 15 minutes during storage, starting at the commencement of the first immediate mixing. Temperature control was based on the assumption that microbial activity would be limited at 4°C, thereby attributing most of the decay due to non-active biological parameters within the matrix, whereas 20°C would include the additional degradation due to microbial activity. (133) Due to the lack of influences on degradation from any pipe biofilms and/or collection tank microbiota from an existing system, this study set out to gauge the stability of the surrogate and/or pathogen in the graywater matrix itself, and is expected to be a conservative estimate of the biological degradation in a real-world system. Sub-samples were processed (100 mL) as described previously, and replicate qPCR assays quantified the decay following 0, 1, 2, 3, 4, 6, and 8 days storage. **Table 13** displays the DNA yields obtained during the storage study.

Figure 15. Image of bioreactor and 556 multi-parameter sensing probe for storage study

Table 13. DNA concentration information for storage study

Replicate	Temp °C	Day	Yield (ng/μL)	260/280
1	4	0	4.7	1.72
		1	5.6	1.66
		2	4.5	1.50
		3	4.3	1.42
		4	3.1	1.57
		6	32.2	1.95
		8	39.5	1.95
	20	1	18.8	1.98
		2	47.9	1.99
		3	49.4	2.05
		4	59.5	1.94
		6	14.3	1.93
		8	62.5	2.01
2	4	0	7.7	1.87
		1	5.3	2.06
		2	6.4	1.82
		3	5.8	2.13
		4	10.7	1.92
		6	48.9	1.94
		8	20.8	1.97
	20	1	24.7	1.95
		2	26.4	1.95
		3	46.4	2.00
		4	36.0	1.90
		6	42.4	1.90
		8	45.1	1.91
3	4	0	5.3	1.69
		1	5.4	1.99
		2	3.7	1.47
		3	3.3	2.08
		4	2.8	1.49
		6	6.6	1.85
		8	38.8	2.01
	20	1	5.2	1.84
		2	19.2	1.90
		3	7.3	2.12
		4	33.8	1.95
		6	27.5	1.97
		8	31.15	1.97

Results

Pilot Study

HmtDNA was detectable for up to 6 days at 4°C and 8 days at 20°C (**Figure 16**). HmtDNA degraded only 16% at 20°C and 21% h at 4°C after 24 hours. At day 3, HmtDNA only exhibited 35% degradation at 4°C and 72% at 20°C. HmtDNA experienced a strong exponential decay curve in the wash at both temperatures with R^2 values greater than 0.9.

Figure 16. HmtDNA decay in pilot study ($R^2 \geq 0.90$).

Pyrosequencing results of all samples in the pilot study are depicted in a stacked bar graph (**Figure 17**). The stack bar graph displays similar signatures after 24 hours of storage at 4°C, suggesting that minimal bacterial change occurred during this time period. At day 3 at 4°C, a significant shift was observed where a majority of the lower-abundant species disappeared. By days 6 and 8, the genus *Massilia* dominated the community. *Pseudomonas* spp. accounted for only a small portion of the reads at day 0, but accounted for over 30% of the reads on day 3 at 4°C and varied throughout the days. At 20°C, an ecological shift in community genera was observed after only 24 hours of storage. During days 3, 6, and 8, *Stenotrophomonas* and

Brevundimonas dominated the community, accounting for approximately 60% of the reads on each day. *Massilia* was also present at 20°C throughout the entire study.

Since sequencing is only semi-quantitative, the read counts were further analyzed to determine any biases in read statistics. At day 0, a total of approximately 12,000 reads were obtained. After one day of storage at 4°C, read counts remained approximately the same (**Figure 18**). However, after one day of storage at 20°C, the total number of reads decreased by approximately 33% to below 8,000 reads per sample. For both temperatures, read counts remained steady between 6,000-8,000 reads per sample for days 3-8.

Figure 18. Pyrosequencing read counts for pilot study

Figure 17. Classification results of Pyrosequencing of stored laundry graywater samples

Storage Study

DO and pH levels dropped to anoxic levels after 24-29 hours at 20°C and 6-7.5 days at 4°C (**Figure 19-20**). A summary of chemical sensor data can be found in **Table 11**. HmtDNA was detectable until day 8 (1.79 ± 0.14) at 4°C, and day 6 (1.08 ± 0.37) at 20°C in stored laundry graywater (**Figure 21**). HmtDNA degraded only 7% and 4% at 20°C and 4°C, respectively after 24 hours (**Figure 22**). HmtDNA experienced a strong first-order exponential decay curve at both temperatures with R^2 values ≥ 0.98 (**Figure 21**). Total *Staphylococcus* rapidly decayed to beyond the detection limit after 24 hours of storage at 20°C (**Figure 23**), degrading 58% in the first 24 hours of storage (**Figure 22**). The opportunistic pathogen *S. aureus* remained detectable in stored laundry graywater for at least 24 hours at 20°C (**Figure 23**) before following the rapid decay pattern of total *Staphylococcus*. At 4°C, total *Staphylococcus* and *S. aureus* appeared to observe no change in concentration above background noise until after day 4 (**Figure 23**) when they plummeted below detection limits, consistent with the drop in DO and pH levels observed around this time period (**Figures 19-20**). A significant decay correlation was observed between HmtDNA and *Staphylococcus* with *S. aureus* at both 4°C and 20°C ($r \geq 0.87$, $P \leq 0.01$).

Figure 19. Laundry graywater storage effect on DO (mg/L) at 4°C and 20°C R = replicate#

Figure 20. Laundry graywater storage effect on pH at 4°C and 20°C (n=3)

Table 14. Initial summary characteristics of 556 sensor data at 4°C and 20°C

Rep.	DO (mg/L)		pH		Specific Conductivity (μS)		Oxidation Reduction Potential (mv)		Salinity (ppt)	
	4°C	20°C	4°C	20°C	4°C	20°C	4°C	20°C	4°C	20°C
1	7.42	7.43	7.89	7.60	631.0	672.0	175.9	161.3	0.31	0.33
2	7.95	6.76	7.87	7.56	620.0	621.0	105.0	255.3	0.30	0.30
3	7.50	8.00	7.91	7.57	510.0	504.0	277.4	562.2	0.25	0.24

Figure 21. Fate of HmtDNA in laundry graywater at 4°C (solid black line) and 20°C (dashed line) (n=3).

Figure 22. Decay of select surrogates and pathogen (*S. aureus*) in laundry graywater at 20°C (n=3)

Figure 23. Fate of total *Staphylococcus* and *S. aureus* in laundry graywater at 4°C ($R^2 = 0.98$) and 20°C (n=3)

Discussion

Pyrosequencing results of the pilot study suggest that little or no significant changes are observed with regards to the microbial quality of the stored laundry graywater after 24 hours at 4°C (**Figure 17**). However, after three days storage at the same temperature, significant changes were observed where certain genera appeared to dominate the matrix. After 24 hours of storage at 20°C, there was an observed change in the detected genera, suggesting that storage of as little as one day can change the microbial quality of laundry graywater. Although chemical measurements such as pH and DO were not taken during the pilot study, they were during the storage study, which could help to explain the shift in the microbial community after one day of storage since a drop in DO and pH were observed around this time period in the storage study (**Figures 19-20**). Due to the semi-quantitative nature of DNA sequencing, it is difficult to determine the significance of sequencing results. A particular bacterial group could have exhibited growth or the DNA of certain genera could have been removed, inactivated, or

destroyed by a number of mechanisms within the system, thereby having less competition available for limited resources during PCR amplification and causing read bias.

The staph species signal disappeared much faster than the HmtDNA signal during the storage study (**Figure 22**). Due to the long persistence of the HmtDNA, it is likely that the HmtDNA present is not naked DNA but rather still inside intact mitochondria. Although the mechanism of degradation and/or disappearance of both surrogates was not a specific focus of this investigation, a number of factors could have contributed to this phenomenon and should be explored in future research. For example, potential factors that might influence the disappearance of the nucleic acids of the staphylococci could be the pH, salinity, detergents/surfactants, chlorine residual, or grazers such as protozoa. Since the pH of skin is approximately 4.5-5.5, (139) and the pH of the laundry graywater was between 7.5-8.0, this could be a cause for destruction of the cell wall and nucleic acids since pH is a major factor in microbial survival. (133) Since various species of staph have shown halophilic tendencies, this water may simply not have the right combination of salts present to sustain internal osmotic balances for microbial processes such as cellular respiration. (131) Additionally, grazers such as protozoa could consume the staph species, but this probably would not account for the full 7-log loss in two days since the source of sufficient amounts of protozoa to enter the water is unclear as evidence from high skin signature identified via sequencing. Instead, the most probabilistic factor is likely to be the surfactants and detergent present in the laundry wash water or the chlorine residual in the influent water that can rupture the gram negative cell wall of the staphylococci species. (133)

One possible approach for future research to determine the disappearance mechanism could be to study a pure culture of a staph species such as *S. aureus* in distilled or de-ionized

water as a control and then manipulate the mixture with several variables. Using this approach, factors such as pH could be adjusted to various acidic or alkaline levels and then the pure culture could be spiked into the matrix and compared to the control of just *S. aureus* and distilled water. The same approach could be used to study the affect of surfactants/detergents, chlorine residual, or various salinity concentrations. Additionally, the larger protozoan grazers could be filtered out with a larger pore size filter (1-5 μ M) prior to the 0.45 μ M filtration; however some original signal of the surrogate may be lost due to particle adherence to suspended particles which could be filtered out by the larger pore size filtration. Since there could be multiple factors affecting the staph signal loss, the exact mechanism should be an area of future research in order to understand the environmental fate of surrogates used for graywater treatment studies.

Previous recommendations suggest that untreated graywater should not be stored for longer than 24-48 hours to avoid aesthetic nuisances, (29, 140) suggesting that both HmtDNA and total *Staphylococcus* could have use as surrogates or indicators in storage applications. The slower decay rates observed for all targets during storage at 4°C when compared to 20°C is likely explained by temperature-dependent enzymatic nucleases, since temperature has previously been identified in influencing enzyme DNA degradation. (141, 142) The apparent relationship between rapid staphylococci degradation with decreased DO and pH levels in stored, untreated laundry graywater indicates that total *Staphylococcus* could be used as an indicator of “fresh” laundry graywater. However, this rapid degradation could become problematic for measuring microbial removal during treatment since any system is likely to require a transient storage tank to equalize flow to the system which could degrade the signal depending on the time stored. In contrast, HmtDNA remained extremely stable after one day of storage at 20°C, decaying by less than 5% during that time period (**Figure 22**) and could potentially just be attributed to PCR noise

and variation. The predictable first order decay rate experienced by HmtDNA ($R^2 \geq 0.98$) at both temperatures suggests that its robustness can provide information about the age and potentially the quality of the graywater. This could be important in reuse systems since some bacterial populations have been shown to grow when untreated graywater is stored for periods of time. (6) HmtDNA also persisted several days longer than the staphylococci bacteria, indicating that it is likely to have a higher concentration to measure a wider dynamic range of pathogen removal during treatment of transiently stored graywater.

The drop in DO observed between 24-29 hours of storage at 20°C is consistent with previous investigations suggesting that aesthetic nuisances (smell) may play a role in governing these systems, since anoxic or anaerobic tend to give off a distinct and unpleasant odor. This data also suggests that microbial activity is taking place in the system during this storage period, actively metabolizing any substrate and releasing CO₂ into the solution as by-products of glycolysis. The CO₂ will react with water to form carbonic acid (H₂CO₃) which could help to explain the drop in pH observed around the same timeframe. However, the more likely culprit for pH drop is the production of volatile fatty acids via fermentation at low DO levels, (133) typical of a solution with no continuous input of nutrients and O₂ to support aerobic growth. The same observations are applicable to the 4°C reactor; however, the anaerobic/anoxic drop takes more time, likely due to slower enzymatic processes at lower temperatures as previously discussed and the fact that cooler water can hold more dissolved O₂. (143) The specific conductivity and oxidation reduction potentials (ORP) observed (**Table 13**) are in the range of reported wastewater parameters between primary and secondary effluent, supporting previous investigations that graywater quality can lie in-between these reported parameters. (18) An ORP around 700mV is typical to achieve significant microbial kill within minutes during chlorine

disinfection, (144) confirming that additional treatment of laundry graywater is likely to be necessary to ensure necessary pathogen reduction. The salinity measured in laundry graywater is in line with surface water salinity reports, (107) which would be expected with the laundering of athletic clothes exposed to sweat.

Chapter 5: Summaries and Conclusions

Previous graywater microbiological research has focused largely on FIB as an indicator of enteric pathogens, (1, 2) and used this information in quantitative microbial risk assessment (QMRA) frameworks to predict risk in graywater. (17) However, this approach is limited due to the inability of FIB to accurately predict risk (2, 17, 19) and the lack of dose-response data for the diversity of potential waterborne pathogens that may be excreted and transmitted via other pathways such as oral/respiratory or dermal, (21) and potentially urinal. (145) Therefore, this investigation set out to provide data on consistent and abundant biological targets found endogenously in the laundry graywater matrix that could be used to evaluate treatment removal efficiencies and monitor pathogenic log reductions.

Pyrosequencing revealed that the most abundant bacterial genera classified in industrial laundry graywater were *Staphylococcus*, *Corynebacterium*, *Micrococcus*, *Lactobacillus*, *Acinetobacter*, and *Propionibacterium* spp., representing over 46% of the reads classified in our study (**Figures 7-8**). The human microbiome project identified these same genera as some of the main inhabitants on human skin, concluding that *Staphylococcus*, *Propionibacterium* and *Corynebacterium* were the most abundant organisms on human skin. (76, 78) Other dominant members such as *Actinobacteria*, *Micrococcus*, and *Lactobacillus* have also been found to be common skin- or human-associated bacteria. (76, 79) Traditional FIB genera such as *Bacteroides* spp. and *Enterococcus* spp. were present but represented less than 9.0×10^{-4} % of classified reads. This data unsurprisingly indicates that the majority of the bacterial load in laundry graywater is of skin and/or human origin.

Although SH samples collected from drain pipes had some overlap with the common skin-associated organisms observed in laundry (e.g., *Propionibacterium*, *Acinetobacter*, *Corynebacterium*, *Staphylococcus*), SH also contained distinct genera prevalent in wastewater

infrastructure such as *Acinetobacter*, *Aeromonas*, *Cloacibacterium*, *Tolumonas*, *Enhydrobacter*, *Pseudomonas*, *Streptococcus*, *Propionivibrio*, *Bacteroides*, (94) and *Dechloromonas* and *Zoogloea*. (95) The similarity in composition between the BC and SH samples suggests that flushing of loose cells or sloughing of biofilms within the collection system strongly influence the composition of collected SH water, even with as little as 1.5m of collection pipes. The importance of building infrastructure bacteria found in the work reported herein is also in alignment with recent research, which found that 81.4% of bacterial sequences in untreated wastewater were related to sewage infrastructure bacteria (i.e. non-fecal). (101) This data suggests that skin-associated organisms are likely to contribute the largest human signal in graywater, particularly graywater that is relatively “fresh” in nature. However, water infrastructure-like organisms are likely to be the most abundant bacteria in water recycling systems and that these genera could potentially give the widest dynamic range for measuring log reduction in reuse scenarios. Future research should be directed at combining various sources of graywater (shower, laundry, sink, etc.) into a matured temporary equalization tank from which samples should be taken to explore the potential microbes that may dominate this habitat and play a key role in these environments immediately prior to treatment.

The qPCR results confirm that low levels of fecal contamination are present in laundry graywater as previously concluded. (2) However, these targets are generally very low in quantity at 0-2 log₁₀ per 100mL and do not accurately predict risks from other potential pathogen shedding pathways such as respiratory or dermal ($P \geq 0.01$ with *S. aureus*). Therefore, FIB should not be used to assess human health risk or pathogen removal in laundry graywater. In contrast, HmtDNA and other skin-associated bacteria such as total *Staphylococcus* were abundant in laundry graywater, generally between 3-7 log₁₀ per 100mL. The higher abundances

of these targets allows for a wider dynamic range by which to measure pathogen log removal in treatment systems designed for water reuse. In addition, both *Staphylococcus* and HmtDNA positively correlated with the opportunistic pathogen *S. aureus* ($P \leq 0.01$) suggesting that these two markers could be indicative of skin-related health risks, but future research must confirm this phenomenon. However, caution should be used with total *Staphylococcus* as a surrogate for treatment if it is desired to transiently store laundry graywater for extended periods of time prior to treatment, due to the fairly quick disappearance of the signal observed in our results. The mechanism of this disappearance should be an area of future research. Instead, total *Staphylococcus* could be used as a marker of “fresh” graywater which could also aid in water quality assessment. In contrast to staphylococci, HmtDNA may be a more attractive alternative to measure pathogen removal in treatment systems when transient storage is applicable due to the abundance, stability and predictable first-order decay rate observed at both 20°C and 4°C.

The endogenous surrogate treatment breakthrough method as an assessment of microbial graywater quality is useful because it eliminates the need to detect a diversity of potential pathogens while simultaneously monitoring treatment performance and risk to reuse. This approach also removes the need for external surrogate spiking and has potential for future online monitoring capabilities. However, in order to achieve a scientifically conservative infection risk of 1:10,000, (146) a minimum of 4-log pathogen removal/inactivation may be necessary as currently used for surface water treatment, (30) which based on the endogenous levels present in un-stored laundry graywater, could be undertaken with HmtDNA or skin-associated organisms such as total *Staphylococcus*, once relationships with specific pathogens by treatment have been identified. Although non-viral surrogate use as a treatment indicator for pathogens such as viruses may be questioned due to differing physical size and structure thereby potentially

influencing behavior, significant treatment correlations have been found between bacterial surrogates (*C. perfringens*) and viruses (enterovirus) in previous water treatment evaluations, (33) suggesting that the proposed surrogates are worthy of consideration in future investigations. Hence, this research identifies HmtDNA and skin-associated bacteria such as total *Staphylococcus* as potential surrogates for use in pathogen risk and removal in laundry graywater reuse treatment systems.

Due to the differing microbial populations found in various types of graywater, it is recommended that future research studying these matrices in different geographical and temporal locations validate our results. Once validated, these microbial markers could be identified for more rigorous research to assess their potential use for endogenously monitoring treatment performance and efficiency. In the meantime, since all graywater types are likely to have a human component that we have shown to contribute a significant amount of human-associated microbes to the matrix, the proposed surrogates such as HmtDNA and *Staphylococcus* should be studied in other matrices to validate the premise that they are present and behave similarly in all types of graywater. In addition, the mechanisms for the signal loss of the staphylococci bacteria should be investigated further to determine the environmental fate factors that may affect a surrogate's effectiveness during treatment. Several potential techniques mentioned previously would be a good start towards understanding these potential influences. Therefore, it is pertinent for future research to study the relationships between the proposed surrogates and potential pathogens of concern to determine if useful treatment relationships are established before acceptance can be warranted.

Appendix A

Table 15. HmtDNA Sanger Sequence Top Matches

#	Sample ID	Top Match	e-value	Accession	Score	max identity
1	2-1p8F-01	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	1.0E-66	KC994161.1	261	99.00%
2	2-1w8F-02	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	4.0E-66	KC994161.1	259	99.00%
3	2-1w11F-03	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	9.0E-68	KC994161.1	265	99.00%
4	2-11p8F-04	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	2.0E-69	KC994161.1	270	99.00%
5	2-11w8F-05	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	4.0E-66	KC994161.1	259	99.00%
6	2-11p11F-06	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	2.0E-64	KC994161.1	254	99.00%
7	2-11w11F-07	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	1.0E-65	KC994161.1	257	99.00%
8	2-13p8F-08	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	5.0E-65	KC994161.1	255	99.00%
9	2-13w8F-09	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	4.0E-66	KC994161.1	259	99.00%
10	2-14p8F-10	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	1.0E-65	KC994161.1	257	99.00%
11	2-14w8F-11	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	2.0E-64	KC994161.1	254	99.00%
12	2-14p11F-12	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	1.0E-65	KC994161.1	257	99.00%

13	2-14w11F-13	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	1.0E-65	KC994161.1	257	99.00%
14	2-18p8F-14	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	3.0E-67	KC994161.1	263	99.00%
15	2-18w8F-15	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	9.0E-63	KC994161.1	248	97.00%
16	2-20p8F-16	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	2.0E-64	KC994161.1	254	99.00%
17	2-20w8F-17	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	2.0E-64	KC994161.1	254	99.00%
18	2-20w11F-18	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	2.0E-66	KC994161.1	261	99.00%
19	2-25p8F-19	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	5.0E-65	KC994161.1	255	99.00%
20	3-4p8F-20	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	1.0E-65	KC994161.1	257	99.00%
21	3-4w8F-21	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	1.0E-65	KC994161.1	257	99.00%
22	3-4p11F-22	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	2.0E-64	KC994161.1	254	99.00%
23	3-18p8F-23	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	1.0E-66	KC994161.1	261	99.00%
24	3-18w8F-24	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	4.0E-66	KC994161.1	259	99.00%
25	3-18p3F-25	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	2.0E-64	KC994161.1	254	99.00%
26	3-18w3F-26	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	1.0E-66	KC994161.1	261	99.00%

27	3-25p8F-27	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	3.0E-68	KC994161.1	267	99.00%
28	3-25w8F-28	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	1.0E-65	KC994161.1	257	99.00%
29	5-13p8F-29	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	4.0E-66	KC994161.1	259	99.00%
30	5-13w8F-30	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	1.0E-65	KC994161.1	257	99.00%
31	5-20p8F-31	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	9.0E-68	KC994161.1	265	99.00%
32	5-20w8F-32	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	1.0E-65	KC994161.1	257	99.00%
33	5-20p3F-33	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	4.0E-66	KC994161.1	259	99.00%
34	5-20w3F-34	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	1.0E-66	KC994161.1	261	99.00%
35	5-23p3F-35	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	1.0E-66	KC994161.1	261	99.00%
36	5-23w3F-36	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	1.0E-65	KC994161.1	257	99.00%
37	5-28p8F-37	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	8.0E-84	KC994161.1	318	99.00%
38	5-28w8F-38	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	1.0E-65	KC994161.1	257	99.00%
39	5-28p3F-39	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	1.0E-65	KC994161.1	257	99.00%
40	5-28w3F-40	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	1.0E-66	KC994161.1	261	99.00%

41	2-1p8R-41	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	5.0E-65	KC994161.1	255	99.00%
42	2-1w8R-42	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	8.0E-63	KC994161.1	248	99.00%
43	2-1w11R-43	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	5.0E-65	KC994161.1	255	99.00%
44	2-11p8R-44	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	6.0E-64	KC994161.1	252	99.00%
45	2-11w8R-45	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	4.0E-71	KC994161.1	276	99.00%
46	2-11p11R-46	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	1.0E-61	KC994161.1	244	99.00%
47	2-11w11R-47	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	1.0E-60	KC994161.1	241	99.00%
48	2-13p8R-48	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	2.0E-69	KC994161.1	270	99.00%
49	2-13w8R-49	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	2.0E-68	KC994161.1	267	99.00%
50	2-14p8R-50	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	2.0E-69	KC994161.1	270	99.00%
51	2-14w8R-51	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	4.0E-71	KC994161.1	276	99.00%
52	2-14p11R-52	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	2.0E-68	KC994161.1	267	99.00%
53	2-14w11R-53	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	2.0E-68	KC994161.1	267	99.00%
54	2-18p8R-54	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	2.0E-69	KC994161.1	270	99.00%

55	2-18w8R-55	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	2.0E-69	KC994161.1	270	99.00%
56	2-20p8R-56	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	2.0E-69	KC994161.1	270	99.00%
57	2-20w8R-57	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	2.0E-69	KC994161.1	270	99.00%
58	2-20w11R-58	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	4.0E-71	KC994161.1	276	99.00%
59	2-25p8R-59	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	4.0E-71	KC994161.1	276	99.00%
60	3-4p8R-60	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	2.0E-69	KC994161.1	270	99.00%
61	3-4w8R-61	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	2.0E-69	KC994161.1	270	99.00%
62	3-4p11R-62	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	2.0E-69	KC994161.1	270	99.00%
63	3-18p8R-63	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	4.0E-71	KC994161.1	276	99.00%
64	3-18w8R-64	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	2.0E-69	KC994161.1	270	99.00%
65	3-18p3R-65	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	1.0E-67	KC994161.1	265	99.00%
66	3-18w3R-66	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	2.0E-70	KC994161.1	274	99.00%
67	3-25p8R-67	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	9.0E-68	KC994161.1	265	99.00%
68	3-25w8R-68	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	7.0E-69	KC994161.1	268	99.00%

69	5-13p8R-69	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	3.0E-72	KC994161.1	279	99.00%
70	5-13w8R-70	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	4.0E-71	KC994161.1	276	99.00%
71	5-20p8R-71	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	3.0E-72	KC994161.1	279	99.00%
72	5-20w8R-72	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	2.0E-68	KC994161.1	267	99.00%
73	5-20p3R-73	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	2.0E-69	KC994161.1	270	99.00%
74	5-20w3R-74	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	9.0E-68	KC994161.1	265	99.00%
75	5-23p3R-75	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	2.0E-68	KC994161.1	267	99.00%
76	5-23w3R-76	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	2.0E-70	KC994161.1	274	99.00%
77	5-28p8R-77	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	2.0E-69	KC994161.1	270	99.00%
78	5-28w8R-78	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	2.0E-69	KC994161.1	270	99.00%
79	5-28p3R-79	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	1.0E-66	KC994161.1	261	99.00%
80	5-28w3R-80	Homo sapiens isolate Mara3710_Maranao mitochondrion, complete genome	3.0E-72	KC994161.1	279	99.00%

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