Greywater reuse: an analysis of microbial performance surrogates applied to ultraviolet irradiation

by

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ABSTRACT

The potential constraints and testing needs surrounding safe greywater reuse are poorly understood. Given the wide variety of contaminants present in different greywater from home to home and even between sources within the same home, there are unique treatment needs and considerations for each. The aim of the work performed in this thesis was to assess these needs and provide an improved way to test greywater treatment performance over current use of coliforms. A literature review was performed, indicating significant variances in faecal indicator bacteria (FIB) across sources, and even within the same sources. While FIB (e.g. faecal coliforms/*E.* coli & enterococci) are useful to verify municipal water treatment, the same cannot be said for greywater. Thus, an alternative target is needed to not only indicate the presence of non-enteric pathogenic organisms present in greywater, but also to be indigenous to greywater at a high enough level to assess at least a four-$\log_{10}$ bacterial treatment reduction. Given the high prevalence of total staphylococci on human skin, total staphylococci were selected as a potential surrogate to represent bacterial reduction when exposed to the most commonly used greywater disinfection process, ultraviolet (UV) irradiation. *Staphylococcus* spp. and select FIB were subjected to UV irradiation; while the potentially pathogenic *Staphylococcus aureus* was found to be less resistant to UV than FIB, other *Staphylococcus* spp., such as *S. haemolyticus* and *S. hominis* were significantly more resistant than *S. aureus* and a range of FIB. UV was used to treat raw hand-rinse water from five participants to assess the efficacy of total staphylococci as an endogenous surrogate to assess bacterial reduction; total staphylococci made up the majority of the culturable bacteria before and after irradiation which suggests it would act as an adequate surrogate. While UV is an attractive and relatively low maintenance disinfection method, there are efficacy and safety considerations. Of particular concern, through cyclic growth and exposure, *S. aureus* can theoretically become enriched in a circulating greywater reuse system, with strains becoming more resistant to UV irradiation. Additionally, given the prevalence of personal care products (PCP) in wastewater, especially in greywater, the efficacy of UV towards *S. aureus* in the presence the sunscreen oxybenzone, was assessed. When present in water at a concentration of 10 $mg.mL^{-1}$, oxybenzone was shown to decrease the efficacy of UV-C by nearly one order of magnitude.

PREFACE

Chapter 3 of this thesis has been published as Shoults, D.C. and Ashbolt, N.J. (2017) Total staphylococci as performance surrogate for greywater treatment. *Environmental Science and Pollution Research 2017 DOI 10.1007/s11356-017-9050-1*. (**Paper I**)

Chapter 4 of this thesis has been published as Shoults, D.C. and Ashbolt, N.J. (2017) UV disinfection of hand-rinse greywater and performance testing using indigenous *Staphylococcus* spp. *Water 2017 DOI 10.3390/w9120963*. (**Paper II**)

Chapter 5 of this thesis is to be submitted as Shoults, D.C. and Ashbolt, N.J. Decreased efficacy of UV inactivation of *Staphylococcus* spp. after cyclic exposure and regrowth: Considerations for greywater reuse systems. (**Paper III**)

Professor Nicholas J. Ashbolt and I designed experimental apparatuses and procedures. I was responsible for data collection and analysis as well as the manuscript composition. Professor Nicholas J. Ashbolt was the supervisory author and was involved with concept formation and manuscript composition and edits. Candis Scott performed VITEK^{TM} strain identifications used for **Paper II**.

dedication

I would like to dedicate this thesis to the billions of people living without clean water and sanitation around the globe. My hope is the work performed here may contribute to a more sustainable future by securing adequate access to water and sanitation for all.

"All we have to decide is what to do with the time that is given to us."

-J.R.R. Tolkien

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF EQUATIONS

LIST OF ACRONYMS

A: Absorbance

ATCC: American Type Culture Collection

Bac: *Bacteroides*

BP3: Benzophenone

BR: Mixed bathroom

BT: Bathtub

CFU: Colony forming units

CWW: Clothes wash water

DI: Deionized

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DW: Dish washer

FC: Faecal coliforms

FIB: Faecal indicator bacteria

F-RNA coliphages: F-specific RNA coliphages

GW: Mixed greywater

hBac: Human-specific *Bacteroides*

HmtDNA: Human mitochondrial deoxyribonucleic acid

HPC: Heterotrophic plate count

K: Kitchen

KS: Kitchen sink

L: Laundry

LT: Laundry trough

MPN: Most probable number

MSA: Mannitol salt agar

PC: Polycarbonate

PCP: Personal care products

PCR: Polymerase chain reaction

PW: Personal washing

qPCR: Quantitative polymerase chain reaction

rRNA: ribosomal ribonucleic acid gene

SD: Standard deviation

SDB: Surfactant-degrading bacteria

SH: Shower

Spp.: Species

Staphylococcus spp.: Total staphylococci

TC: Total coliforms

TSA: Tryptic soy agar

TSB: Tryptic soy broth

TS-MSA: Estimated total staphylococci CFU counts on MSA

TSS: Total suspended solids

T-TSA: Total TSA CFU counts

UV: Ultra violet

WB: Washbasin

WHO: World Health Organization

WM: Clothes washing machine

WWTP: Waste water treatment plant

CHAPTER 1: INTRODUCTION AND BACKGROUND

The motivation for this research was to address non-potable household water needs, given the cost and increasing scarcity of suitable freshwater for many rapidly urbanizing regions (Schiermeier 2014), even within developed countries (WHO 2016). Of particular concern are the many circumpolar communities in North America and globally which lack adequate quantities of water for potable and non-potable purposes (Daley *et al.* 2014, Hennessy and Bressler 2016). There is also a greater awareness in the water-energy nexus, and that most household water use does not need to be treated to drinking water quality (Sathe 2013). One option to provide more sustainable water services is to utilize treated greywater (Schoen *et al.* 2014). Greywater, which can be defined as domestic household wastewater without input from the toilet (Ottosson 2003), is a valuable commodity which should be utilized to reduce water usage. In-home greywater reuse is not widely practiced, and is illegal to reuse within homes throughout the majority of North America (NRC 2016); this is a particular limitation for many circumpolar communities still lacking sustainable water and sanitation (Daley *et al.* 2015, Thomas *et al.* 2016).

Current available technologies are capable of effectively treating greywater to potable quality, however the cost/energy use of such systems is high (Cobacho *et al.* 2012) and not necessary for uses such as toilet flushing and clothes washing. There is a need for cost effective and robust greywater treatment systems which can handle the variability of contaminant composition within greywater and produce safe, disinfected water fit for household uses ranging from toilet flushing to laundry, and potentially semi-continuous recirculation of shower water. Overarching any change in community water use is the need for regulatory guidelines to be designed for in-home greywater reuse; including identifying adequate performance surrogates for greywater treatment processes to assess required pathogen log-reductions in the evolving risk-based guidelines (Sharvelle *et al.* 2016). Given the complexities/costs in undertaking controlled spiking studies (Zimmerman *et al.* 2016), the focus of this research was to assess the potential for indigenous *Staphylococcus* spp. (total staphylococci) as a greywater treatment performance surrogate, demonstrated for ultraviolet (UV) irradiation performance testing.

MICROBIAL COMPOSITION OF GREYWATER IN LITERATURE

Depending on the end use of treated greywater, necessary $\log_{10}$ reductions may range from 5 to 13 for viruses, 4 to 9 for *Cryptosporidium*, 3 to 8 for *Giardia*, and 3 to 8 for enteric bacteria

(Schoen *et al.* 2017). In general, the reduction of pathogens of interest can be estimated by the viability or presence of target organisms after treatment. It is important to understand microbial concentrations of FIB, potential performance surrogates, and pathogens of interest in greywater. However, determining the microbiological contamination in greywater can be difficult; each source of greywater presents different potential biological contaminants and concentrations (Birks and Hills 2007) and the microbiome of raw greywater is poorly understood (Callewaert *et al.* 2015). The majority of studies focus on traditional faecal indicators and do not assess the presence of saprozoic and opportunistic skin pathogens (such as *P. aeruginosa* and *S. aureus*). To better understand the variety of contamination between sources, a literature review was performed on 41 studies describing the microbial composition of greywater from an array of different greywater sources. Summarising the data was difficult due to the inconsistency of the reporting of the biological quality between studies. The majority of papers only presented data for faecal coliforms (FC), total coliforms (TC), and *Escherichia coli*, with a variety of other bacteria sometimes reported. A further confounding factor was the media/method used and reporting statistic (mean, median, ranges, *etc*.); most studies used culture-based methods (reported as colony forming units [CFU] or most probably number [MPN]), while a few sources reported assays based on the quantitative polymerase chain reaction (qPCR). Overall, the inconsistency in bacteria assayed, assay methods, and reporting methods as well as the relatively low amount of greywater studies when compared to municipal wastewater makes it difficult to fully understand the key microbial concentrations within greywater.

Of the 41 studies reviewed, only two studies analyzed raw greywater samples for *Staphylococcus* spp. (Benami *et al.* 2016, Zimmerman *et al.* 2014) and eight for *S. aureus* (see Table A.1) (Benami *et al.* 2016, Burrows *et al.* 1991, Casanova *et al.* 2001, Gilboa and Friedler 2007, Kim *et al.* 2009, Maimon *et al.* 2014, Siegrist 1977, Zimmerman *et al.* 2014). *S. aureus* has been shown to colonize approximately 30 to 40 % of humans (Kluytmans *et al.* 1997) while *Staphylococcus* spp. has been shown to dominate the skin microbiota of approximately 60 % of humans (Callewaert *et al.* 2013). While *S. aureus* is a potential pathogen of concern (Ashbolt 2015b, Willyard 2017), especially with the emergence of methicillin-resistant *S. aureus* (MRSA) (Fogarty *et al.* 2015), it is important to assess greywater quality for more than just *S. aureus* to represent the presence of possible opportunistic skin-related pathogens, as it is unknown if it is an index of other skin-related pathogens.

Throughout the studies reviewed, a combined total of 22 targets were identified: Heterotrophic plate count bacteria (HPC), TC, FC, *E. coli*, *Enterococcus* spp., faecal streptococci, *Clostridium perfringens*, total *Bacteroides* spp. (Bac), human-specific *Bacteroides* (hBac), *Staphylococcus* spp., *Corynebacterium* spp., *Propionibacterium* spp., *Pseudomonas* spp., *Salmonella* spp., *S. aureus*, *Pseudomonas aeruginosa*, *Salmonella enterica* Typhimurium, *Klebsiella pneumoniae*, surfactant-degrading bacteria (SDB), *Candida albicans*, coliphages, and human mitochondrial DNA (HmtDNA). The examined studies sourced greywater from bathtubs (BT), mixed bathroom (BR), dishwasher (DW), mixed greywater (GW), mixed kitchen (K), kitchen sink (KS), laundry (L), laundry troughs (LT), personal washing (PW), showers (SH), washbasins (WB), and clothes washing machines (WM). Table 1.1 describes the range of averages reported for bathroom, laundry, kitchen, and mixed greywater sources. See Appendix A for a more in-depth account of the reported microbial concentrations in greywater. All values in Table 1.1 and Appendix A are reported as $\log_{10}$ units per 100 mL; units are in CFU, MPN, or gene copies.

Greywater differs from municipal wastewater in the variance of microbial concentrations found, especially when assessing FIB; as can be seen in Table 1.1, the concentrations of bacteria, especially traditional FIB (TC, FC, *E. coli*, and enterococci) are highly variable between studies. However, due to the lack of data, it is difficult to assess the variance of non-enteric bacteria (such as *Staphylococcus*, *Corynebacterium*, *Propionibacterium*, and *Pseudomonas* spp.).

Table 1.1: Ranges of microbial constituent averages found in different greywater sources

Constituent	Greywater source (CFU/MPN/gene copy per 100 mL)			
	Bathroom	Laundry	Kitchen	Mixed Greywater
TC	2.0 – 8.0 [1]	1.7 – 7.9 [2]	7.3 – 7.9 [3]	1.6 – 8.7 [4]
FC	1.5 – 6.6 [5]	1.1 – 6.6 [6]	4.8 – 6.1 [7]	1.6 – 8.2 [8]
E. coli	1 – 6.4 [9]	1.0 – 6.2 [10]	0 – 5.7 [11]	3.3 – 6.7 [12]
Enterococci	3.4 – 5.5 [13]	2 [14]	-	4.4 – 7.0 [15]
Streptococci	1.0 – 3.3 [16]	1.9 – 2.3 [17]	-	2.4 – 3.2 [18]
HPC	7.2 – 9.3 [19]	-	-	5.8 – 9.2 [20]
S. aureus	4.0 [21]	1.7 [22]	-	ND – 3.7 [23]
P. aeruginosa	ND – 3.5 [24]	-	-	2.5 – 4.3 [25]
***Salmonella* spp.**	5.3 [26]	5.8 [26]	-	4.9 [27]
***Staphylococcus* spp.**	2.7 [28]	6.5 [14]	-	-
Coliphage	ND [29]	-	-	ND – 3.3 [30]
***Corynebacterium* spp.**	-	5.7 [14]	-	-
***Propionibacterium* spp.**	-	5.4 [14]	-	-
HtmDNA	-	2.8[14]	-	-
C. perfringens	0.7 [29]	-	-	3.3 [31]
SDB	-	-	-	4.4 [32]
***Pseudomonas* spp.**	-	4.3 [14]	-	-
S. typhimurium	-	-	-	3.7 [33]
Bac	-	3.3 [14]	-	-
hBac	-	2.7 [14]	-	-
K. pneumoniae	1.4 [28]	-	-	-
C. albicans	ND [34]	-	-	-

Note: data presented in this table is only represented by averages reported in literature. Ranges, minimum and maximum values, and standard deviations were not included in this table. See Appendix A for more data. Values reported as $\log_{10}$ units per 100 mL, with units being CFU, MPN, or gene copies.

[1] (Birks and Hills 2007, Halalsheh *et al.* 2008, Jamrah *et al.* 2006, Jefferson *et al.* 2004, Katukiza *et al.* 2014, Pidou *et al.* 2008, Rose *et al.* 1991, Siegrist *et al.* 1976, Surendran and Wheatley 1998)

[2] (Jamrah *et al.* 2006, Katukiza *et al.* 2014, Siegrist *et al.* 1976, Surendran and Wheatley 1998)

[3] (Brandes 1978, Halalsheh *et al.* 2008, Katukiza *et al.* 2014)

[4] (Brandes 1978, Casanova *et al.* 2001, Gerba *et al.* 1995, Halalsheh *et al.* 2008, Jamrah *et al.* 2006, Jefferson *et al.* 2004, Kim *et al.* 2009, Mandal *et al.* 2011, Ottoson and Stenström 2003, Paris and Schlapp 2010, Pidou *et al.* 2008, Rose *et al.* 1991, Surendran and Wheatley 1998)

[5] (Friedler 2004, Gilboa and Friedler 2007, Halalsheh *et al.* 2008, Jamrah *et al.* 2006, Rose *et al.* 1991, Siegrist *et al.* 1976, Surendran and Wheatley 1998)

[6] (Friedler 2004, Gross *et al.* 2008, Jamrah *et al.* 2006, Rose *et al.* 1991, Siegrist *et al.* 1976, Surendran and Wheatley 1998)

[7] (Brandes 1978, Friedler 2004, Halalsheh *et al.* 2008)

[8] (Brandes 1978, Casanova *et al.* 2001, Dallas *et al.* 2004, Friedler 2004, Friedler *et al.* 2005, Gerba *et al.* 1995, Gross *et al.* 2005, Gross *et al.* 2008, Halalsheh *et al.* 2008, Jamrah *et al.* 2006, Mandal *et al.* 2011, Paris and Schlapp 2010, Rose *et al.* 1991, Surendran and Wheatley 1998)

[9] (Benami *et al.* 2016, Birks and Hills 2007, Chaillou *et al.* 2010, Halalsheh *et al.* 2008, Jefferson *et al.* 2004, Katukiza *et al.* 2014, O'Toole *et al.* 2012, Pidou *et al.* 2008)

[10] (Katukiza *et al.* 2014, O'Toole *et al.* 2012, Zimmerman *et al.* 2014)

[11] (Halalsheh *et al.* 2008, Katukiza *et al.* 2014)

[12] (Halalsheh *et al.* 2008, Kim *et al.* 2009, Mandal *et al.* 2011, Paulo *et al.* 2009)

[13] (Birks and Hills 2007, Chaillou *et al.* 2010, Pidou *et al.* 2008)

[14] (Zimmerman *et al.* 2014)

[15] (Casanova *et al.* 2001, Ottoson and Stenström 2003)

[16] (Jefferson *et al.* 2004, Siegrist *et al.* 1976)

[17] (Siegrist *et al.* 1976)

[18] (Casanova *et al.* 2001, Jefferson *et al.* 2004)

[19] (Friedler *et al.* 2008, Gilboa and Friedler 2007)

[20] (Casanova *et al.* 2001, Friedler *et al.* 2005, Kim *et al.* 2009, Mandal *et al.* 2011)

[21] (Gilboa and Friedler 2007)

[22] (Zimmerman *et al.* 2014)

[23] (Casanova *et al.* 2001, Kim *et al.* 2009, Maimon *et al.* 2014, Siegrist 1977)

[24] (Benami *et al.* 2016, Burrows *et al.* 1991, Gilboa and Friedler 2007)

[25] (Casanova *et al.* 2001, Maimon *et al.* 2014)

[26] (Katukiza *et al.* 2014)

[27] (Mandal *et al.* 2011)

[28] (Benami *et al.* 2016)

[29] (Gilboa and Friedler 2007)

[30] (Casanova *et al.* 2001, Ottoson and Stenström 2003)

[31] (Ottoson and Stenström 2003)

[32] (Gross *et al.* 2007)

[33] (Kim *et al.* 2009)

[34] (Burrows *et al.* 1991)

ANALYSIS OF MICROBIAL INDICATORS FOR GREYWATER REUSE

Indicator organisms and performance surrogates have different applications in water treatment. As stated in **Error! Reference source not found.**, index organisms are used to test for the presence of target pathogens, while performance surrogates are used to assess the efficacy of a treatment process to remove a class of pathogen. While FIB are still relevant to assess the presence of potentially pathogenic enteric pathogens, there is a need for indigenous surrogates to represent the reduction of opportunistic and saprozoic pathogens (such as *S. aureus* and *P. aeruginosa*) which may be present in greywater. Ideally, there are a number of criteria sought when assessing performance surrogates (Busta *et al.* 2003, Nieminski *et al.* 2000, Rice *et al.* 1996, Sinclair *et al.* 2012):

- Naturally occurring (indigenous) in consistently high concentrations; (Criteria 1)
- Inactivation characteristics consistent with target pathogens; (Criteria 2)
- Easily enumerated using rapid, sensitive, and inexpensive assays; (Criteria 3) and
- Do not grow within the system (either in greywater or in biofilms). (Criteria 4)

John Snow's discovery that cholera was a waterborne disease (Snow 1855) and Robert Koch's confirmation (Koch's postulates) to identify waterborne disease agents (Koch 1884) have appropriately raised awareness of the risks associated with faecally contaminated waters (Ashbolt 2015a). However, the sole focus on enteric pathogens (in water) has drawn attention away from other potential pathogens of concern, including overlooked opportunistic and saprozoic pathogens (Ashbolt 2015a), including *P. aeruginosa*, *S. aureus*, *Acinetobacter baumannii*, *Arcobacter butzleri*, *Helicobacter pylori,* non-tuberculous mycobacteria, and several others (Ashbolt 2015b). In 2017, WHO released a document ranking drug-resistant bacteria based on threat to human health; *Acinetobacter baumannii*, *P. aeruginosa*, *Enterobacteriaceae*, *Enterococcus faecium*, *S. aureus*, *Helicobacter pylori*, *Campylobacter* spp., Salmonellae, *Neisseria gonorrhoeae*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Shigella* spp. (Willyard 2017). While not all of these pathogenic bacteria may be present in greywater, *S. aureus*, *P. aeruginosa*, *Salmonellae*, and *S. pneumoniae* may be present and in relatively high concentrations (see Table 1.1).

Traditional Faecal Indicator Bacteria (FIB)

Estimating the risk of greywater reuse and determining measures for treatment performance testing at an individual household level presents difficulties which are not necessarily experienced when treating large quantities of wastewater such as would be found at municipal wastewater treatment plants (WWTP). As suggested by O'Toole *et al.* (2012), while municipal wastewater typically has consistent levels of indicator *E. coli* and enteric viruses (and therefore less variability in the ratio between the two), this is not always the case for greywater at a household level. While FIB and index organisms may be representative of potential faecal pathogens in greywater, they are not always indicative of how pathogens of interest will behave when stressed (such as by UV-C irradiation, chlorine disinfection, washing machine cycle, *etc*.). As an example, a study of laundry greywater by Gerba (2001) reported total $\log_{10}$ reductions of 4.6 and > 7.1 for *S. aureus* and *E. coli* throughout the laundry cycle, respectively, while the enteric adenovirus and hepatitis A virus exhibited $\log_{10}$ reductions between 3 to 4 during the same cycle with the addition of bleach.

Faecal indicator bacteria do not necessarily correspond to the presence of pathogens (Birks and Hills 2007, O'Toole *et al.* 2012). O'Toole *et al.* (2012) showed no significant relationship between indicator *E. coli* and enteric viruses from clothes machine wash water. In addition, they also reported no significant relationship between the presence of underwear in the cloth-wash water (CWW) and indicator *E. coli* ($P = 0.460$), while there was a significant relationship between the presence of underwear and enteric viruses ($P = 0.042$) (O'Toole *et al.* 2012). The lack of relationship could be caused by an extreme reduction in *E. coli* throughout the wash cycle (accounting for the samples positive for pathogenic viruses but negative for *E. coli*), and a low sensitivity of virus detection relative to *E. coli* detection (accounting for samples positive for *E. coli* but negative for pathogenic viruses) (O'Toole *et al.* 2012). Furthermore, none of the FIB assayed in the Zimmerman *et al.* (2014) study showed any significant correlation with each other or to HmtDNA, suggesting an inadequacy for indicating the presence of enteric or skin related pathogens.

The greywater microbial assessment by O'Toole *et al.* (2012) showed a significant difference in viable indicator *E. coli* cells between the water from wash and rinse cycles (mean $\log_{10}$ values of 5.0 and 3.5). The explanation of reduction of *E. coli* in the laundry cycle is further supported by a

laundry greywater study by Gerba (2001) that exhibited total $\log_{10}$ reductions of 4.6 and > 7.1 for *S. aureus* and *E. coli* throughout the laundry cycle, respectively. Zimmerman *et al.* (2014) also reported 50% and 40% detection of *E. coli* in rinse and wash cycles, which is consistent with O'Toole *et al.* (2012).

A study by Gross *et al.* (2008) reported that the mean concentration of FC in laundry greywater was more than 2-$\log_{10}$ higher than the median value; Gross *et al.* (2008) suggested *"average values are often not representative when considering treatment of small volumes such as from a single household."* Many of the studies reviewed reported consistently higher means of FIB in raw greywater when compared to median values (see Appendix A); this is due to the high number of non-detects and low-detects of FIB. An emphasis must be placed on non-enteric pathogens for the assessment of risks associated with greywater reuse, as well as the testing for treatment performance required to reduce pathogens associated with GW reuse.

Some of the FIB may exhibit growth within greywater systems (*e.g. E. coli*) (Ottosson and Stenström 2003). If regrowth occurs either in the raw greywater holding tank or after treatment as described in Friedler and Gilboa (2010), it is problematic to quantify the reduction occurring across any treatment step. Furthermore, the relatively low concentrations of traditional indicator organisms are not adequate surrogates to represent the log-reductions likely required to produce safe greywater (Gilboa and Friedler 2007, Sharvelle *et al.* 2016).

Staphylococcus spp.

Staphylococcus spp. are a prevalent Gram-positive skin bacterial group, which have been shown to dominate the skin microbiota of people (Callewaert *et al.* 2013). As shown in Table A.1, Zimmerman *et al.* (2014) found total staphylococci to be the most prevalent bacteria in laundry water; moreover, *Staphylococcus* spp. were detected in all samples, with significant correlations ($\alpha \leq 0.01$) to *Propionibacterium*, *Enterococcus*, *Pseudomonas* (which may include the pathogenic *P. aeruginosa*), hBac, *E. coli*, HmtDNA, and *S. aureus*. Consistent with the Zimmerman *et al.* (2014) findings was the study by Benami *et al.* (2016) which reported total staphylococci consistently more prevalent in raw greywater and at higher concentrations than the FIB *E. coli*. Though the study by Callewaert *et al.* (2015) reported less consistent detection of total staphylococci than when compared with Zimmerman *et al.* (2014) and Benami *et al.* (2016), *Staphylococcus* spp. was in the top two of relative abundance of Gram-positive bacteria in

samples along with *Propionibacterium*. Equally as important as its high prevalence in raw greywater, is staphylococci's inherent resistance to stress environments such as a washing and drying laundry cycles; A study by Munk *et al.* (2001) showed that when compared to Gram-negative bacteria (*E. coli* and *P. aeruginosa*), *S. aureus* and *S. epidermidis* showed significantly higher resistance throughout the wash cycle. Munk *et al.* also showed that 100 % of *E. coli* and *P. aeruginosa* were killed after being exposed to a wash cycle at 50 ^{o}C, while *S. aureus* and *S. epidermidis* survived the same cycle, however were killed (100 %) at 60 ^{o}C (Munk *et al.* 2001); these results are consistent with the findings from the aforementioned study by Gerba (2001) indicating greater survival of *S. aureus* in the laundry cycle when compared to *E. coli* (log_{10} reductions of 4.6 and > 7.1, respectively).

Corynebacterium, Propionibacterium, Micrococcus and *Pseudomonas* spp.

When assayed in greywater studies, *Corynebacterium*, *Propionibacterium*, *Micrococcus* and *Pseudomonas* spp. were found to be at consistently high concentrations in greywater (Benami *et al.* 2016, Keely *et al.* 2015, Zimmerman *et al.* 2014). Keely *et al.* (2015) reported *Corynebacterium* spp. to be more abundant than *Staphylococcus* spp. in laundry samples, *Propionibacterium* and *Pseudomonas* spp. more abundant than *Staphylococcus* spp. in shower, equalization tank, and building control samples. Given the high prevalence of these four genera in greywater it is conceivable for any of these four genera to be considered as endogenous surrogates for pathogen reduction, however, there are further considerations. *Pseudomonas* spp., which are Gram-negative and thinner cell walled than the Gram-positive *Staphylococcus*, *Corynebacterium*, *Propionibacterium*, and *Micrococcus* spp. makes the former considerably more vulnerable to UV irradiation than *S. aureus* and *E. coli*, amongst other organisms (Abshire and Dunton 1981, Simonson *et al.* 1990, Zhang *et al.* 2015). Additionally, *Propionibacterium* and *Pseudomonas* have been shown to grow biofilms in surfaces and elsewhere within greywater systems (Achermann *et al.* 2014, Bédard *et al.* 2016, Callewaert *et al.* 2015, Friedler and Gilboa 2010). While it is still possible these genera may be adequate surrogates for greywater treatment, these options are not evaluated further in this study.

ULTRAVIOLET IRRADIATION OF MICROORGANISMS IN WATER

UV irradiation was the disinfection process of interest in this thesis due to its relatively low environmental impacts (compared to chemical disinfection processes), ease of use for small

systems, and relatively small space requirements (U.S. EPA 2003). The lack of residual effects which may otherwise be left with chlorine disinfection make UV irradiation an attractive process for decentralized, user-operated water treatment systems. However, there are limitations and research gaps pertaining to the use of UV irradiation for greywater treatment systems; there is a need to better understand the fate of pathogens, indicators, and potential surrogates when exposed to UV irradiation (NRC 2012). UV irradiation is highly effective in inactivating most bacteria and *Cryptosporidium* and *Giardia* (oo)cycsts; however, some viruses and bacterial spores are less effected (Chang *et al.* 1985, Hijnen *et al.* 2006). Less studied is the fate of helminths and fungal spores when exposed to UV irradiation and other disinfection processes. Recent studies have shown helminths and fungal spores to be extremely resistant to UV irradiation when compared to most bacteria (Brownell and Nelson 2006, Wen *et al.* 2017); helminths may require UV doses of four times that of the UV resistant adenovirus for a 1-$\log_{10}$ reduction (Brownell and Nelson 2006) and fungal spores have been exhibited UV resistance in the range of more resistant bacteria and some viruses (Wen *et al.* 2017). There is a collective need for a numerical relationship for UV reduction between bacteria, viruses, protozoa, fungi, and helminths to be understood and compiled, and for the relationships between performance surrogates and respective pathogens be understood (Ju *et al.* 2016, Rudko *et al.* 2017).

GREYWATER REUSE GUIDELINES

While greywater reuse is illegal in the majority of the developed world, some countries are pioneering the reuse of reclaimed greywater and developing guidance documents. Two of the more developed documents on the forefront of reclaimed water guidelines are the *Australian Guidelines for Water Recycling* (NRMMC *et al.* 2006), and WE&RF's *Risk-Based Framework for the Development of Public Health Guidance for Decentralized Non-Potable Water Systems* (Sharvelle *et al.* 2017). Although Canada does have a set of guidelines for reclaimed greywater reuse, the document includes little detail on the log reduction credits necessary from different greywater sources, and pertains only to toilet/urinal flush use of reclaimed wastewater (Health Canada 2010). Additionally, some provinces within Canada (e.g. British Columbia) do have guidelines for greywater reuse, however such documents are incomplete and lack pertinent information on risk management and performance testing (BC Ministry of Environment 2013). Most guidelines focus on the use of FIB and do not recognize the importance for risk-based performance testing (Health Canada 2010, U.S. EPA 2012). A 2012 document released by the

National Research Council (NRC 2012) listed several key research needs for reclaimed water reuse, including the need identify and understand better indicators and surrogates for process performance monitoring, and a better understanding or pathogen removal efficiencies; the intent of the work performed in this thesis was to in part address the these needs.

CHAPTER 2: OBJECTIVES AND SCOPE

The study by Zimmerman *et al.* (2014) was one of the first studies to take a molecular approach to assessing the microbial composition of greywater. Their findings of the abundance of *Staphylococcus* spp. partially set the precedent for the material studied in this thesis. Zimmerman *et al.* (2014) showed that FIB are not reliably capable of also acting as an endogenous surrogate for pathogen reduction in greywater systems due to their relatively low and inconsistent concentrations greywater samples (Criteria 1[*]), relatively low resistance to stress environments (Criteria 2), and their ability to grow within greywater systems (Criteria 4). Little is known as to the efficacy of UV inactivation towards staphylococci, and in particular the pathogenic *S. aureus* (Benami *et al.* 2013). Overall, there is a collective need for action-spectra and dose-response curves describing UV inactivation of *Staphylococcus* spp. generally and within greywater. Furthermore, given the above listed limitations with FIBs as process indicators for the removal of pathogens in greywater reuse, knowledge on the efficacy of UV inactivation towards staphylococci may provide future options for risk management monitoring. Most limiting is a lack of knowledge on the fate of pathogens (both enteric and saprozoic) via different greywater treatment systems (Ashbolt 2015a, National Research Council of the National Academics (NRC) 2012). If demonstrated of value for enteric bacteria performance, total staphylococci may also provide value to address these additional pathogen concerns. Furthermore, additional considerations to efficacy and safety of UV irradiation of greywater are explored in the following chapters, thus the overall aims of this thesis are as follows:

1. Assess the efficacy of total staphylococci as an endogenous surrogate for treatment performance testing of greywater systems (Chapters 3 & 4);
2. Assess the role of PCP compounds on the efficacy of UV irradiation (Chapter 3);
3. Compare variance of UV resistance of different *Staphylococcus* spp. (Chapter 4); and
4. Determine if certain pathogenic bacteria may become enriched and increase in UV resistance within a greywater system using UV irradiation (Chapter 5).

[*] Performance surrogate criteria can be found in Chapter 1.

CHAPTER 3: TOTAL STAPHYLOCOCCI AS PERFORMANCE SURROGATE FOR GREYWATER

Adapted from *Environmental Science and Pollution Research DOI 10.1007/s11356-017-9050-1* (Shoults and Ashbolt 2017a)

ABSTRACT

Faecal indicator bacteria (FIB) are commonly used as water quality indicators; implying faecal contamination and therefore the potential presence of pathogenic, enteric bacteria, viruses, and protozoa. Hence in wastewater treatment the most commonly used treatment process measures (surrogates) are total coliforms, faecal coliforms, *Escherichia coli* (*E. coli*), and enterococci. However, greywater potentially contains skin pathogens unrelated to faecal load, and *E. coli* and other FIB may grow within greywater unrelated to pathogens. Overall, FIB occur at fluctuating and relatively low concentrations compared to other endogenous greywater bacteria; affecting their ability as surrogates for pathogen reduction. Therefore, unlike for municipal sewage, FIB provide a very limited and unreliable log-reduction surrogate measure for on-site greywater treatment systems. Based on a recent metagenomic study of laundry greywater, skin-associated bacteria such as *Staphylococcus*, *Corynebacterium* and *Propionibacterium* spp. dominate, and may result in more consistent treatment surrogates than traditional FIB. Here we investigated various *Staphylococcus* spp. as potential surrogates to reliably assay over 4-$\log_{10}$ reduction by the final-stage UV disinfection step commonly used for on-site greywater reuse, and compare them to various FIB/phage surrogates. A collimated UV beam was used to determine the efficacy of UV inactivation (255 nm, 265 nm, and 285 nm) against *E. coli*, *Enterococcus faecalis*, *E. faecium*, *E. casseliflavus*, *Staphylococcus aureus*, and *S. epidermidis*. *Staphylococcus* spp. was estimated by combining the bi-linear dose-response curves for *S. aureus* and *S. epidermidis*, and was shown to be less resistant to UV irradiation than the other surrogates examined. Hence a relative inactivation credit is suggested, whereas the doses required to achieve a 4 and 5-$\log_{10}$ reduction of *Staphylococcus* spp. (13.0 mJ.cm^{-2} and 20.9 mJ.cm^{-2} respectively) were used to determine the relative inactivation of the other microorganisms investigated. The doses required to achieve a 4 and 5-$\log_{10}$ reduction of *Staphylococcus* spp. resulted in a $\log_{10}$ reduction of 1.4 and 4.1 for *E. coli*, 0.8 and 2.8 for *E. faecalis*, 0.8 and 3.6 for *E. casseliflavus*, and 0.8 and 1.2 for MS2 coliphage, respectively. Given the concentration difference of *Staphylococcus* spp. and FIB (3 to 5-$\log_{10}$ higher), we propose the use of *Staphylococcus* spp. as a novel endogenous performance surrogate to demonstrate greywater treatment performance given its relatively high and consistent concentration and therefore ability to demonstrate over 5-$\log_{10}$ reductions.

Key words: Greywater, *Staphylococcus*, staphylococci, water treatment, indicator organisms, surrogates, UV disinfection, collimated beam

INTRODUCTION

Available freshwater is an increasingly scarce commodity for many rapidly urbanizing regions, even within developed countries (WHO 2016). However, increasing population growth in relatively water-scarce regions along with an increase in personal water consumption have greatly contributed to the urban water deficit faced around the world (Schiermeier 2014). There is also a greater awareness in the water-energy nexus (Sathe 2013), and that most household water use does not need to be treated to drinking water quality. In particular, many circumpolar communities lack adequate quantities of water for potable and non-potable purposes (Daley *et al.* 2014, Hennessy and Bressler 2016). Hence, one option to provide more sustainable water services is to utilize treated greywater (Schoen *et al.* 2014). Greywater, which can be defined as domestic household wastewater without input from the toilet (Ottosson 2003), is a valuable commodity which should be utilized to reduce water usage. In-home greywater reuse is not widely practiced, and is illegal to reuse within homes throughout the majority of North America (NRC 2016), where there are many circumpolar communities still lacking sustainable water and sanitation (Daley *et al.* 2015, Thomas *et al.* 2016).

Current available technologies are capable of effectively treating greywater to potable quality; however the cost of such systems is high (Cobacho *et al.* 2012) and not necessary for uses such as toilet flushing and clothes washing. There is a need for a cost effective and robust greywater treatment system that can handle the variability of contaminant composition within greywater and produce safe, disinfected, non-potable water for households uses ranging from toilet flushing to laundry, and potentially semi-continuous recirculation of shower water. Additionally, there is a need for regulatory guidelines to be designed for in-home greywater reuse including identifying adequate performance surrogates for greywater treatment processes to assess required pathogen log-reductions in risk-based guidelines (Sharvelle *et al.* 2016). Depending on the end use of the treated greywater, necessary $\log_{10}$ reductions may range from 5 to 13 for viruses, 4 to 9 for *Cryptosporidium*, 3 to 8 for *Giardia*, and 3 to 8 for bacteria (Schoen *et al.* 2017). Given complexities/costs in undertaking controlled spiking studies (Zimmerman *et al.* 2016), here we present the potential for using endogenous *Staphylococcus* spp. (total staphylococci) as a

greywater treatment performance surrogate, demonstrated for Ultraviolet (UV) irradiation performance testing.

Disinfection is an essential process in water treatment to remove/inactivate pathogenic organisms. UV inactivation is a commonly used disinfection treatment method in both wastewater and drinking water treatment, which directly damages the nucleic acids of microorganisms and inhibits future replication (Gross *et al.* 2015). UV is an attractive disinfection method and considered to be a more environmentally friendly disinfection technology than chemical disinfection (Winward *et al.* 2008). Additionally, UV is often preferred over chlorine because the use of chlorine may leave residual chlorine compounds that may have adverse effects, such as generating odorous substances and biohazardous disinfection by-products (Chang *et al.* 1985, Mori *et al.* 2007). However, UV irradiation does have its limitations; Winward *et al.* (2008) showed raw greywater having higher turbidity, larger mean particle size, lower UV_{254} transmittance, and higher total suspended solids (TSS) levels relative to raw municipal wastewater. This can be explained by the lack of dilution of greywater when compared to municipal wastewater. Winward *et al.* (2008) also showed that the presence of larger particles in greywater limited the effectiveness of UV irradiation, causing an extreme tailing effect of coliforms reduction even in with UV doses up to 239 $mJ.cm^{-2}$. Hence, for UV irradiation to be an effective pathogen reduction step in greywater treatment, adequate pre-treatment must be performed to ensure adequate UV_{254} transmissivity and removal of larger particles capable of shielding microorganisms.

Process Indicators, Faecal Indicators, and Index Organisms

There is often confusion between the semantics and purposes of process indicators (surrogates), faecal indicators, and pathogen index organisms. It is important to make a distinction between the indicators and surrogates, as the roles of such are not necessarily interchangeable. Table 3.1 exhibits the key differences between the three.

Table 3.1: Definitions for indicator and index microorganisms of public health concern. Adapted from World Health Organization (2001, 2016).

Indicator Group	Definition
Process indicator (surrogate)	A group or organism that demonstrates the efficacy of a process, such as total heterotrophic bacteria or total coliforms for chlorine disinfection, and F-RNA coliphages as models of human enteric virus behaviour.
Faecal indicator	A group or organism that indicates the presence of faecal contamination, such as the bacterial groups thermotolerant coliforms/E. coli or enterococci; hence, they only infer that pathogens may be present.
Pathogen Index	A group/or species indicative of pathogen presence, such as E. coli as an index for Salmonella presence, and qPCR Norovirus for human enteric viruses

Process indicators (surrogates) and faecal indicator bacteria (FIB) are commonly used as indicators in water treatment to determine the potential presence of enteric viruses, bacteria, and parasitic protozoan pathogens that maybe associated with faecal contamination (U.S. EPA 2012). The most commonly tested indicators are total coliforms, faecal coliforms, *Escherichia coli*, and enterococci (U.S. EPA 2012). However, most greywater treatment studies inappropriately utilize FIB as an indicator of pathogen risk (Ottosson and Stenström 2003). Determining the microbiological contamination in greywater can be difficult; each source of greywater presents different potential biological contaminants and concentrations (Birks and Hills 2007). Zimmerman *et al.* (2014) investigated the 16S rRNA diversity of laundry water and identified skin-associated bacterial members of *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* as the major members of that microbiome; using quantitative polymerase chain reaction (qPCR) they showed that in the university gym laundry samples taken, *Staphylococcus* spp. averaged from 3 to 5 orders of magnitude higher than faecal source markers including total *Bacteroides* spp., human-specific *Bacteroides*, *Enterococcus* spp., and *E. coli*, in ascending orders of magnitude, respectively (Zimmerman *et al.* 2014). Due to the direct contact of clothing with human skin and the prevalence of the opportunistic pathogen *Staphylococcus aureus* on the human body (Zimmerman *et al.* 2014), *S. aureus* has also been measured in greywater, typically at concentrations up to 5 x 10^5 cfu.100mL^{-1} (Burrows *et al.* 1991, Gilboa and Friedler 2007, Nolde 1999), but as with any pathogen, is not always present. Hence, *S. aureus,* enteric

pathogens and FIB are present in varying and often low concentrations in greywater, with some of the FIB exhibiting growth within greywater systems (e.g. *E. coli*) (Ottosson and Stenström 2003). If regrowth occurs either in the raw greywater holding tank or after treatment as exhibited in Friedler and Gilboa (2010), it is problematic to quantify the reduction occurring across any treatment step. Furthermore, traditional indicator organisms used in North America are not adequate surrogates to represent the log-reductions likely required to produce safe greywater (Gilboa and Friedler 2007, Sharvelle *et al.* 2016). Hence, the focus of this paper is to determine if endemic greywater staphylococci, including pathogens such as *S. aureus*, may be suitable treatment surrogates to reliably assay over 4-$\log_{10}$ reductions of key pathogens (Birks and Hills 2007, Fogarty *et al.* 2015, Gross *et al.* 2007, Zimmerman *et al.* 2014) by UV disinfection.

Ultraviolet Irradiation of Staphylococci

Commercial UV systems generally deliver 254 nm UV-C from mercury-vapour lamps that impact on nucleic acid within microorganisms; however, some manufacturers also use polychromatic UV-C lamps (including higher wavelength UV-C) that also impact on cellular proteins (Eischeid and Linden 2011). Little is known as to the efficacy of UV inactivation towards staphylococci, and in particular the pathogenic species *S. aureus* (Benami *et al.* 2013). Overall, there is a collective need for action-spectra and dose-response curves describing UV inactivation of S. a*ureus*, as well as other *Staphylococcus* spp. generally and within greywater. Furthermore, given the above listed limitations with FIBs as process indicators for the removal of pathogens in greywater reuse, knowledge on the efficacy of UV inactivation towards staphylococci may provide future options for risk management monitoring. Most limiting is a lack of knowledge on the fate of pathogens (both enteric and saprozoic) via different greywater treatment systems (Ashbolt 2015a); if demonstrated of value for enteric bacteria performance, total staphylococci may provide value to address these additional pathogen concerns.

MATERIALS AND METHODS

Bacterial Culturing, Plating, and Enumeration

Freeze dried *S. aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), *Escherichia coli* (ATCC 13115), *E. coli** (ATCC 15597), *Enterococcus faecalis* (ATCC 29212), and *Enterococcus casseliflavus* (ATCC 9199) were purchased from American Type Culture Collection (ATCC) and revived according to ATCC protocols. ATCC bacteria culture stocks

were stored at -80 ^{o}C in a cryomedium to glycerol ratio of 3:2. When needed, bacteria cultures were inoculated into the brain heart infusion (BHI) liquid media and incubated at 37 ^{o}C.

Phage Culturing, Plating, and Enumeration

Aliquots (200µL) of the F-RNA coliphages MS2 (ATCC 15597-B1) were used to infect *E. coli** while in its exponential phase during incubation as described in Method 1601 (U.S. EPA 2001). Briefly, after 18 hours of growth and infection time, the sample was filtered through a 0.22µm filter to recover the coliphages and plaque within a semi-soft tryptic soy agar (TSA) overlay containing 0.7% agar. Plates were then incubated at 37^{o}C for 24 hours before counting plaque-forming units (PFUs).

UV Irradiation

Liquid medium containing the test organism was diluted to 1:100 (for bacteria) and 1:1000 (for MS2 coliphage) in sterile deionized (DI) water inside a 60 mm Petri plate containing a 5 mm x 2 mm stir bar which was then placed on a magnetic stirrer at 400 rpm to facilitate mixing without a vortex forming. DI water was used rather than tap water in order to minimize potential contact with residual chlorine, which could have an inactivation effect on the cells (Zyara *et al.* 2016). Controls with bacteria suspended in sterile DI water were performed to investigate any loss in viability due to osmotic pressure change. No significant difference in $\log_{10}$ CFU count was observed from 100 seconds after pipetting from the BHI liquid media (the time needed to dilute the cells to a countable dilution) to 20 minutes (the estimated maximum time cells might be suspended in DI water throughout the experiment). Prior to exposing the sample to the UV, a 100 µL sample was pipetted out of the mixed solution and diluted to the appropriate dilution before being plated in triplicate. The collimated beam was then placed over the sample and the desired wavelength dose was delivered to the sample.

Based on an initial experiment performed for each test organism to determine the approximate dose needed for a 4-$\log_{10}$ reduction, a second experiment was performed to estimate an 11-point dose-response curve. Samples were taken at 0 %, 15 %, 30 %, 45 %, 60 %, 75 %, 90 %, 105 %, 120 %, 135 %, 150 % of the dose calculated for an estimated 4-$\log_{10}$ reduction. Subsamples (100 µL) were taken at appropriate times, diluted in DI water, and plated to determine the $\log_{10}$ reduction at each dosage using the following equation:

$$\text{Log}_{10}\, Reduction = Log\left(\frac{\frac{CFU\ Control}{100mL}}{\frac{CFU\ Dosage}{100mL}}\right) \quad \text{Equation 3.1}$$

An AquaSense Pearl Beam collimated UV reactor (Florence, KY USA) was used to deliver 255nm, 265nm or 285nm UV-C irradiation to test organisms suspended in water using a modified EPA protocol (U.S. EPA 2006). Equation 3.2 was used to calculate the effective intensity (E_{ave}) of the collimated beam based on measurable variables (NSF International 2014):

$$E_{ave} = 0.98\left[\frac{E_0}{L}\left(\frac{(1-A)^L-1}{ln(1-A)}\right)\right] \quad \text{Equation 3.2}$$

The incident intensity (E_0) was measured using a NSF certified radiometer (UVP Radiometer, Model UVX-25). The water height (L) was measured to 1 cm (28.3 mL in a 60mm cylindrical Petri dish), and a spectrophometer (Thermo Scientific Genesys 10S UV-VIS) was used to measure the absorbance (A) of 255nm UV by the suspending medium. The resulting E_{ave} was then multiplied by the exposure time (seconds) in order to calculate the resulting dosage measured in $mJ.cm^{-2}$.

RESULTS

Data points from each experiment were plotted to display the dose-response of each organism relative to 255nm UV irradiation. Figure 3.1 shows the relative dose-response to UV by *S. aureus*, *S. epidermidis*, *E. faecalis*, *E. casseliflavus*, *E. coli* and MS2 coliphage.

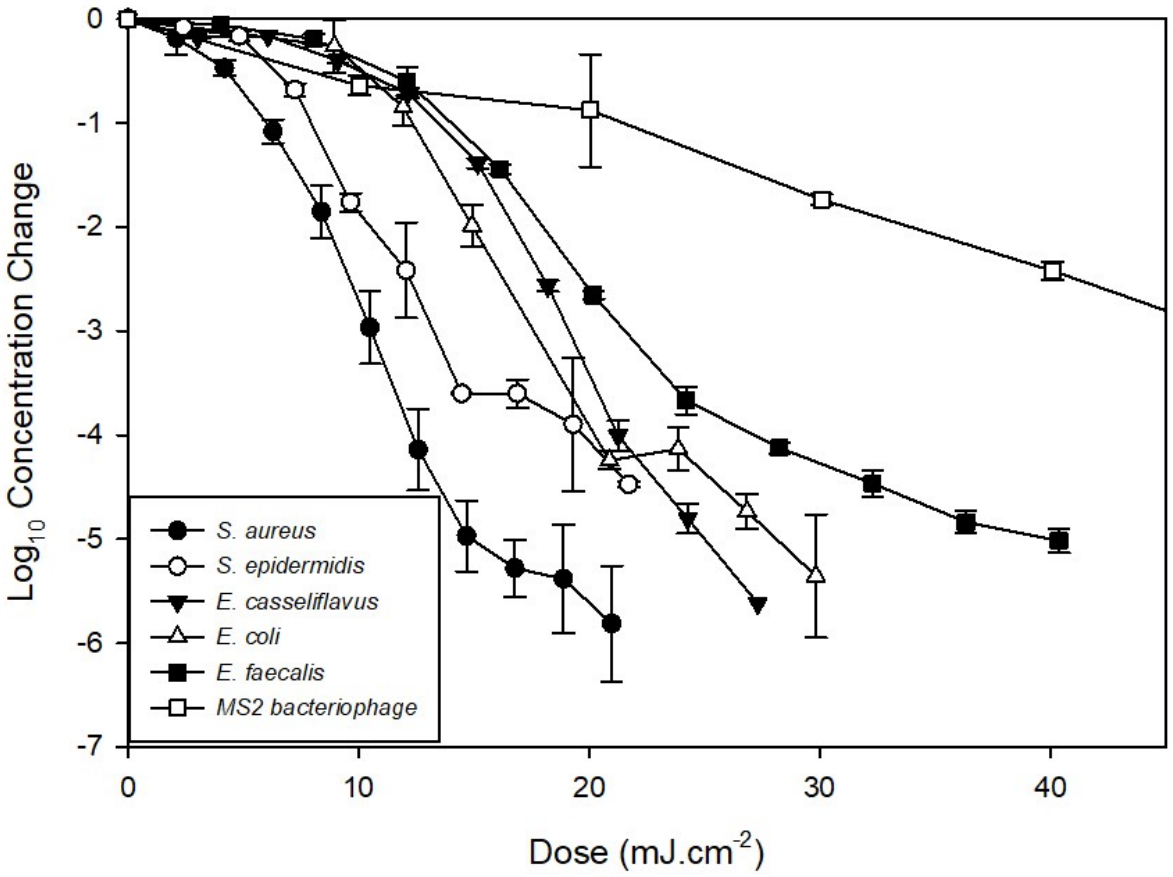

Figure 3.1: Comparison of inactivation of *Staphylococcus* spp., FIB, and MS2 bacteriophage by 255nm UV (average log reduction values ± SD)

S. aureus and *S. epidermidis* are generally the most common *Staphylococcus* spp. colonizing humans (Coates *et al.* 2014), and therefore were used in order to represent total staphylococci in the bench experiments to determine the efficacy of UV inactivation. Data points for the *S. epidermidis* decay curve were interpolated to estimate $\log_{10}$ reduction values for the dosages for each *S. aureus* point. A weighted average was then taken of each point to estimate a dose-response curve for *Staphylococcus* spp., which is shown in Figure 3.2.

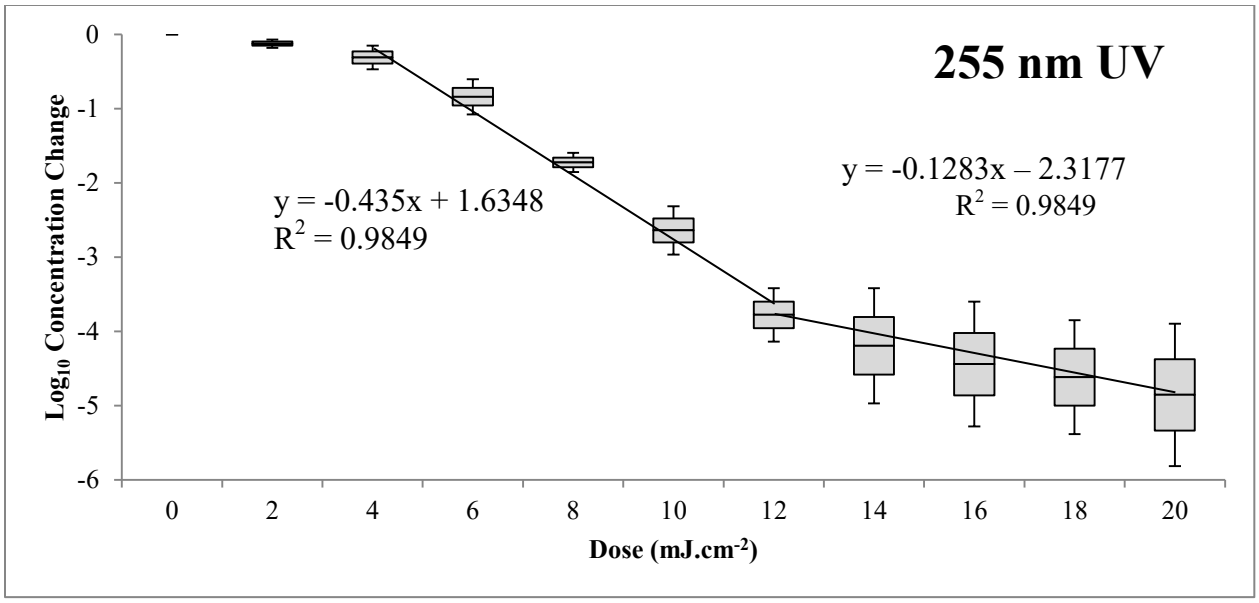

Figure 3.2: Bi-linear box and whisker plot for UV (255nm) decay curve of total staphylococci (average of S. aureus and S. epidermidis)

Figure 3.2 also depicts the bi-linear decay curve with total staphylococci; two trend lines were plotted to determine the decay equation (in units of $\log_{10}$ reduction [LR] per $mJ.cm^{-2}$) in the bi-linear graph to determine the decay coefficients K1 (0.44 LR per mJ.cm-2) and K2 (0.13 LR per $mJ.cm^{-2}$). These equations, along with the decay equations and respective R^2 values for the other test organisms are shown in Table 3.2. These equations were used to determine the relative reduction for each organism compared to total staphylococci.

Table 3.2: 255nm UV decay curves for various organisms (linear equations represent the line of best fit for the linear segments of each decay curve)

Organism	**Linear equation (x units: [$mJ.cm^{-2}$])**	**R^2**
***Staphylococcus* spp.**	y = -0.44x + 1.6 (x < 12)	0.98
	y = -0.13x - 2.3 (12 < x < 20)	0.97
E. coli	y = -0.34x + 3.0 (9 < x < 21)	0.99
	y = -0.13x + 1.3 (21 < 30)	0.83
E. faecalis	y = -0.26x + 2.6 (12 < x < 24)	1.0
	y = -0.085x - 1.7 (24 < x < 40)	0.98
E. casseliflavus	y = -0.35x + 3.8 (14 < x < 25)	0.98
MS2 coliphage	y = -0.061x + 0.032 (0 < x < 90)	0.99

Comparison of 255nm, 265nm, and 285nm UV on *S. aureus* Inactivation

Also explored was a comparative evaluation of 255 nm, 265 nm, and 285 nm UV irradiation on *S. aureus* to determine the most effective wavelength for inactivation. The initial experiment for 285nm yielded very little inactivation and was not explored any further. The 255 nm (11.8 $mJ.cm^{-2}$ for 4-log_{10} reduction) UV wavelength was observed to be more effective in inactivating *S. aureus* than 265nm (17.1 $mJ.cm^{-2}$ for a 4-log_{10} reduction).

DISCUSSION

Previously published data on FIB identified them as poor performance surrogates for greywater treatment (Birks and Hills 2007, Ottosson and Stenström 2003, Zimmerman *et al.* 2014). Key criteria for a suitable performance surrogate are given in Table 3.3**,** which compares FIB and *Staphylococcus* spp. Traditional FIB failed to satisfy the first three criteria, due to their inconsistent presence with varying and generally low concentration (Ottosson and Stenström 2003, Zimmerman *et al.* 2014) and lack of correlation with pathogen presence (Birks and Hills 2007); this is enough to determine that traditional FIB are not suitable performance surrogates for greywater treatment. In contrast, total staphylococci meet the first three criteria due to their consistent and high concentration in greywater (Burrows *et al.* 1991, Casanova *et al.* 2001, Gilboa and Friedler 2007, Nolde 1999, Zimmerman *et al.* 2014); presumably, due to the high presence of staphylococci colonizing human skin (Coates *et al.* 2014) and the correlation of presence when human mitochondrial DNA (HmtDNA) is detected in greywater (Zimmerman *et al.* 2014). It is important to acknowledge that although some studies showed a non-detect of *S. aureus* in raw greywater samples (Casanova *et al.* 2001, Siegrist 1977) this is no surprise for a pathogen (as we are not always infected), whereas other skin staphylococci are likely to be present. For example, Zimmerman *et al.* (2014) found *S. aureus* to be approximately of 5-log_{10} lower concentration than *Staphylococcus* spp. in laundry greywater. In addition, approximately 30-40 % of humans carry *S. aureus* (Cole *et al.* 2001, Kluytmans *et al.* 1997), while *Staphylococcus* spp. has been shown to dominate the microbiota of approximately 60 % of humans (Callewaert *et al.* 2013). The fourth criteria explored, stating that an appropriate performance surrogate must have greater survival than target pathogens is still in question. More research is needed to determine the spectrum of genera present within a variety of greywater sources and their treatment requirements for a 3.5 to 6-log_{10} removal, as likely needed for different domestic greywater reuse (Sharvelle *et al.* 2016). Although *Staphylococcus* spp.

showed less resilience to UV than the FIB bacteria tested in the collimated UV beam bench test, total staphylococci are expected at a minimum of 3-$\log_{10}$ higher in concentration than the FIB traditionally used. Due to the high and consistent concentrations of *Staphylococcus* spp., we are suggesting inactivation curves for each reference pathogen can be used to determine $\log_{10}$ reductions relative to *Staphylococcus* spp. (WHO 2016).

Table 3.3: Comparison of FIB and *Staphylococcus* spp.

Criteria	**FIB**	***Staphylococcus* spp.**
Consistent presence	✖	✓
Higher concentration than target pathogens	✖	✓
Identifiable correlation to presence of target pathogens	✖	✓
Same or greater survival as target pathogens	?	?

Since total staphylococci were observed to be more susceptible to inactivation by UV irradiation than FIB, *Staphylococcus* spp. inactivation cannot be used as enteric pathogen surrogates, such as *E. coli*, enterococci and MS2 coliphage. In order to gauge the relative $\log_{10}$ reduction between S*taphylococcus* spp. and the other organisms which were tested, the linear equations for each organism was used to estimate the $\log_{10}$ reduction that would be observed relative to the dosage required for a 4 and 5 $\log_{10}$ reduction for *Staphylococcus* spp., being 13.0 mJ.cm^{-2} and 20.9 mJ.cm^{-2} respectively. These dosages were then used in the linear equations obtained from each decay curve (displayed in Table 3.2) to determine their relative $\log_{10}$ reduction to *Staphylococcus* spp. (Table 3.4).

Table 3.4: Log_{10} reduction credits relative to 4 and 5-$\log_{10}$ reduction of *Staphylococcus* spp.

Organism	**Log_{10} reduction observed at 13.0 mJ.cm^{-2}**	**Log_{10} reduction observed at 20.9 mJ.cm^{-2}**
***Staphylococcus* spp.**	4	5
E. coli	1.4	4.1
E. faecalis	0.8	2.8
E. casseliflavus	0.8	3.6
MS2 coliphage	0.8	1.2

Given the high concentrations of *Staphylococcus* spp. found in greywater relative to FIB (ranging from 3 to 5-$\log_{10}$ higher) (Zimmerman *et al.* 2014), total staphylococci may still serve as a conservative measure for pathogen reduction for enteric bacteria, especially at the dosage for an observed 5-$\log_{10}$ reduction of *Staphylococcus* spp.; the $\log_{10}$ reduction difference at 20.9 mJ.cm^{-2} observed between *Staphylococcus* spp. and the enteric bacteria tested is less than 3-$\log_{10}$, which is satisfactory given the 3 to 5-$\log_{10}$ concentration difference reported by Zimmerman *et al.* (2014). However, the UV dosage required to achieve a 5-$\log_{10}$ reduction of *Staphylococcus* spp. is estimated to only achieve a ~1.2-$\log_{10}$ reduction in MS2 coliphage; this suggests *Staphylococcus* spp. would likely not be an adequate surrogate for enteric virus reduction in greywater, and an additional surrogate, such as endogenous (staphylococci) bacteriophages, is needed to represent enteric virus reduction.

CHAPTER 4: UV DISINFECTION OF HAND-RINSE GREYWATER AND INDIGENOUS *STAPHYLOCOCCUS* SPP.

Adapted from *Water DOI 10.3390/w9120963* (Shoults and Ashbolt 2017b)

ABSTRACT

Greywater reuse is a feasible solution for decreasing raw water extraction in urban and rural settings. However, pathogen-specific performance guidelines and regulations have only recently been recommended, but practical means to assess performance are missing. Here we examine the efficacy of *Staphylococcus* spp. as an endogenous surrogate for greywater pathogen reduction performance testing, by evaluating UV-C irradiation of hand-rinse greywater, and the variability in UV resistance between different wild *Staphylococcus* species. Hand-rinse greywater samples were collected from five participants, and a collimated UV-C beam (256 nm) was used to assess $\log_{10}$ reductions. Assays of colony-forming units on tryptic soy agar (TSA) were compared to mannitol salt agar (MSA) using Lysostaphin™ to confirm *Staphylococcus* spp. After irradiating raw hand-rinse samples to a dose of 220 mJ.cm^{-2}, $\log_{10}$ reductions of *Staphylococcus* spp. were similar (2.1 and 2.2, respectively, $P = 0.112$). The similarity of the reduction based on TSA and *Staphylococcus*-specific culture assays following UV irradiation and the dominating presence of *Staphylococcus* spp. suggests that *Staphylococcus* spp. could be used as an endogenous performance surrogate group for greywater treatment testing. Suspended wild *Staphylococcus* isolates were irradiated with 256 nm UV-C to compare the variability of different *Staphylococcus* species. *Staphylococcus* isolates exhibited significant variance in $\log_{10}$ reduction values when exposed to 11 mJ.cm^{-2} of UV-C. *Staphylococcus hominis* subsp. *hominis* exhibited surprising resistance to UV-C, with only a 1.6 $\log_{10}$ reduction when exposed to 11 mJ.cm^{-2} of UV-C (most other isolates exhibited > 5 $\log_{10}$ reduction). The efficacy of UV-C was also significantly reduced when the sunscreen oxybenzone was present at a possible endogenous greywater concentration.

Keywords: greywater; water reuse; greywater reuse; *Staphylococcus aureus*, *Staphylococcus* spp.; alternative water sources; onsite treatment and reuse; sustainable urban water use

INTRODUCTION

According to UN Water, approximately 2 billion people globally live in areas of water scarcity and another 1.6 billion face economic water shortage (lacking the necessary infrastructure for water transportation) (UN Water 2007). Current municipal water distribution practices of treating all wastewater (blackwater and greywater combined) are generally neither economic nor sustainable for future generations (Chang *et al.* 2012, Strengers and Maller 2012). Greywater is typically defined as household wastewater without faecal contribution (*e.g.* toilet effluent); this includes sources such as wash-basin, shower/bath, laundry, *etc*. The practice of greywater reuse is a relatively unexplored concept in urban developments; it has the potential to reduce municipal water demands by 50 % on average (NRC 2016) and up to 70 % (Ashbolt 2011) depending on the end use. However, in many parts of the world, including North America, the practice of domestic greywater reuse is in general illegal (NRC 2016). An important step forward in gaining government endorsement for greywater reuse is a better understanding of the contaminants, treatment needs, and appropriate treatment system performance testing, as required in water safety plans (WHO 2006).

This paper is a follow-up to a previous study by Shoults and Ashbolt (2017a) to better understand how to assess ultraviolet (UV) irradiation performance testing without the need for externally spiked surrogates. The original study was based off a next-generation sequencing study by Zimmerman *et al.* (2014) which identified *Staphylococcus* spp. as the most abundant bacterial genus in laundry greywater sourced from a university sports facility. To better understand the microbiological constituents of greywater, a literature review was conducted and identified 41 studies, however, only three studies measured greywater for total staphylococci (Benami *et al.* 2016, Keely *et al.* 2015, Zimmerman *et al.* 2014), with several focusing on *Staphylococcus aureus* (Burrows *et al.* 1991, Casanova *et al.* 2001, Gilboa and Friedler 2007, Gross *et al.* 2007, Keely *et al.* 2015, Kim *et al.* 2009, Maimon *et al.* 2014, Zimmerman *et al.* 2014). Of the three studies that measured total staphylococci, total staphylococci were found to be among the most abundant bacteria when compared to faecal coliforms (FC), total coliforms (TC), *Escherichia coli*, *Enterococcus* spp., and other traditional faecal indicator bacteria (FIB). Given the pathogenic nature of *S. aureus* (Arikawa *et al.* 2002, Ramsey *et al.* 2016), which along with *S. epidermidis* is among the most prevalent species on human skin (Coates *et al.* 2014), we proposed total staphylococci be considered as an endogenous surrogate to represent performance

testing in greywater treatment systems, given the inherent problems with using FIB (Shoults and Ashbolt 2017a). Overall, there is a collective need for the scientific community and regulatory bodies to better understand the efficacy of prospective surrogates to assess greywater treatment performance. The experiments in the current study explored an array of considerations to determine the efficacy of total staphylococci as an endogenous performance surrogate.

As with any disinfection process, there are limitations to UV irradiation. While the effects of large suspended particles on the efficacy of UV are understood (Winward *et al.* 2008), the role of micro-pollutants and personal care products (PCP) on the efficacy of UV irradiation are relatively unknown. Given the high reported concentrations of the sunscreen benzophenone (BP3), commonly referred to as oxybenzone (Ramos *et al.* 2016), the effects of oxybenzone on the efficacy of UV irradiation for staphylococci was also studied.

MATERIALS AND METHODS

Hand-Rinse Samples to Recover Skin-Bacteria and Evaluate Their UV-Resistance

Hand-rinse samples were collected from five participants using a "glove method". Participants inserted each hand, one at a time, into a large powderless latex free nitrile glove filled with 40 mL of municipally sourced, sterile deionized (DI) water. Using their free hand, participants massaged the submerged hand to ensure maximum water to hand contact for bacterial shedding. After approximately 30 seconds of exposure, participants inserted their other hand and repeated the previous step. Sterile DI water was used instead of tap water to reduce background bacterial input and confounding effects of residual chlorine. Soap was not used so as to reduce potential bacteriostatic effects and to avoid increased turbidity, as the goal was not to simulate greywater production, but rather to isolate skin-borne bacteria for UV-irradiation and enumerate viable cells using two isolation agars. The resulting bacterial suspension was poured into a sterile beaker and thoroughly mixed. A 28.3 mL portion of the sample was poured into a 60 mm sterile Petri dish so as to give a 1 cm greywater depth. A sterile 5 mm x 2 mm stir bar was placed in the dish and the dish was placed onto a magnetic mixer operating at 400 rpm to facilitate mixing without vortexing.

An AquaSense Pearl Beam collimated LED UV reactor (Florence, KY USA) with a peak wavelength of 256 nm and a half bandwidth of 11.5 nm was used to deliver 256 nm UV-C

irradiation to the raw hand-rinse sample using an adapted EPA protocol (U.S. EPA 2006). Equation 4.1 was used to calculate the effective intensity (E_{ave}) of the collimated beam based on measurable variables (NSF International 2014):

$$E_{ave} = 0.98\left[\frac{E_0}{L}\left(\frac{(1-A)^L-1}{ln(1-A)}\right)\right] \quad \text{Equation 4.1}$$

The incident intensity (E_0) was measured using a NSF certified radiometer (UVP Radiometer, Model UVX-25, Upland, USA). The water height (L) was measured to 1 cm (28.3 mL in a 60 mm cylindrical Petri dish), and a spectrophometer (Thermo Scientific, Genesys 10S UV-VIS, Waltham, USA) was used to measure the absorbance (A) of 256 nm UV in the suspension. The resulting E_{ave} was then multiplied by the exposure time (seconds) in order to calculate the resulting dosage in $mJ.cm^{-2}$.

To estimate staphylococci counts prior to irradiating the sample, 100 µL of the sample was diluted to 10^{-1}; 100 µL of the dilution was pipetted into 15 mL of sterile 0.85 % NaCl buffer then filtered through a 60 mm diameter filter cup apparatus with a 0.22 µm polycarbonate (PC) membrane filter (Isopore™ GTTP-04700, Cork, IRL) using a vacuum pump. The filtering process was performed a total of six times and the resulting filter papers were placed on tryptic soy agar (TSA) and mannitol salt agar (MSA) plates, both in triplicate.

For UV irradiation of samples, the collimated beam was placed over the Petri dish containing 28.3 mL of a raw hand-rinse sample (as described above), and then irradiated to a dose of 220 $mJ.cm^{-2}$. In order to achieve a consistent dose for each person's greywater sample, the absorbance was measured prior to exposure to adjust the exposure time for a resulting dose of 220 $mJ.cm^{-2}$. After exposing samples to 220 $mJ.cm^{-2}$, the entire sample was poured into a sterile 50 mL capped test tube and vortexed to ensure adequate mixing. Upon vortexing, 1 mL of the post-irradiated sample was pipetted into 15 mL of sterile 0.85 % NaCl buffer and plated using the above described filter-plating technique. TSA and MSA plates were incubated at 37 ºC for 18-24 hours before assaying colony forming units (CFU).

Given the similarity in growth conditions of the expected staphylococci and micrococci and that no selective medium is known to easily resolve these genera, we confirmed pure isolates using Lysostaphin™ tablets, known to reliably differentiate between staphylococci (lysis) and

micrococci (no lysis) (Ewald 1986). Upon assaying, Lysostaphin™ tablets were used for total staphylococci confirmation for colonies from MSA plates (Hardy Diagnostics 2017). Five Lysostaphin™ confirmations were performed on each of the triplicate MSA plates, for a total of 30 confirmations per sample (15 at 0 mJ.cm^{-2} and 15 at 220 mJ.cm^{-2}). The fraction of lysis positive Lysostaphin™ confirmation tests was multiplied by the CFU counts from the MSA assays to estimate the fraction of CFU assayed from MSA which were considered totally staphylococci. Equation 4.2 was used to calculate the $\log_{10}$ reduction after exposure for each assay:

$$\text{Log}_{10}\, Reduction = Log\left(\frac{\frac{CFU\ Control}{100mL}}{\frac{CFU\ Dosage}{100mL}}\right) \qquad \text{Equation 4.2}$$

Hand-Rinse Isolates

Upon assessing the MSA control plates, two or three random colonies from each participant were isolated onto separate MSA plates, which were then incubated for 18-24 h at 37 ºC, and then re-streaked at least once more onto MSA plates to ensure purity. Once pure cultures were isolated, they were analyzed using a VITEK™ (2 COMPACT) instrument to determine genera and species.

The collimated UV beam procedure was performed on a total of 14 isolates from five different participants as well as a clinical *S. aureus* strain acquired from the American Type Culture Collection (ATCC), *S. aureus* (ATCC 25923). A presumed *Staphylococcus epidermidis* (ATCC 12228) strain was identified as *Staphylococcus lentus* upon VITEK™ confirmation and also used in this study. Overnight cultures grown in Tryptic Soy Broth (TSB) were diluted to 1:100 in a sterile 0.85 % NaCl buffer. Once diluted, the above collimated UV beam procedure was performed to irradiate suspended cells (with the following modification). Prior to irradiation, 100 µL of the sample was plated on TSA plates in triplicate at the appropriate dilution to effectively assay control plates. Samples were irradiated to 11 mJ.cm^{-2} (slightly less than the approximate dose required for ~4-$\log_{10}$ reduction of *S. aureus*, being a likely targeted reduction level) (Shoults and Ashbolt 2017a), and allowing for post exposure detection of at least some samples. Upon irradiation, 100 µL of the sample was plated in triplicate on TSA at the appropriate dilutions to effectively assay the plates, and $\log_{10}$ reductions were calculated using

Equation 4.2. A portion of the samples were irradiated at 7, 9, and 11 $mJ.cm^{-2}$ in order to estimate decay equations.

Oxybenzone (BP3)

The collimated UV beam procedure described above was performed using *S. aureus* (ATCC 25923) to determine the effects of a sunscreen, benzophenone (BP3), commonly referred to as oxybenzone, on the performance of UV inactivation. Oxybenzone was used as a test compound due to the relatively high concentrations (reports as high as 0.7 $mg.L^{-1}$) when compared to other UV filters/sunscreens (Ramos *et al.* 2016). Since the source of oxybenzone in municipal wastewater is likely from greywater, oxybenzone and other micro-pollutant/PCP concentrations would likely be higher in greywater than in municipal wastewater (which is diluted by blackwater). As shown in a study by Palmquist and Hanæus (2005), organic compound pollutants were typically in greater concentrations within greywater samples when compared to blackwater and sometimes an order of magnitude higher in concentration. Therefore an oxybenzone concentration range of 1 $mg.L^{-1}$ to 10 $mg.L^{-1}$ was used due to the expected increase of oxybenzone concentration in greywater. Oxybenzone has been reported in wastewater effluents at concentrations as high as 0.7 $mg.L^{-1}$ (Ramos *et al.* 2016). An oxybenzone concentration range of 1 $mg.L^{-1}$ to 10 $mg.L^{-1}$ was used due to the expected increase of oxybenzone concentration in greywater when compared to municipal wastewater (which includes blackwater). Oxybenzone was dissolved into dimethyl sulfoxide (DMSO) (166.7 $\mu L.L^{-1}$) and pipetted into sterile 0.85 % NaCl buffer at two different concentrations: 10 mg oxybenzone.L^{-1} and 1 mg oxybenzone.L^{-1}; DMSO was suspended into two separate buffer solutions (at 166.7 & 16.67 $\mu L.L^{-1}$ for the two solutions respectively) used as controls to adjust for any confounding effects of DMSO on the reduction of *S. aureus* by UV irradiation. Overnight cultures of *S. aureus* (ATCC 25923) suspended in TSB were suspended into the four samples (10 mg oxybenzone.L^{-1}, 1 mg oxybenzone.L^{-1}, and respective controls) and irradiated to a UV-C dose of 11.8 $mJ.cm^{-2}$ using the previously described collimated beam apparatus protocol. Samples were assayed on TSA plates in at least triplicate prior to and after exposure to UV. Log_{10} reductions were quantified using Equation 4.2. A paired t-test was performed between oxybenzone and respective controls on the log_{10} reduction means.

Control Experiments

Two additional control experiments were performed to ensure homogeneity with literature as well as consistency throughout the experiments. An 11-point dose-response curve using MS2 bacteriophage (ATCC 15597-B1) was performed using a double agar protocol and assayed by plaquing (EPA 2001); this curve was then compared to a UV-C study by Liu and Zhang (2006) performed using MS2 and was found to be within the same $\log_{10}$ reduction range (see Figure B.1 for graph comparison).

Statistical Analysis

All statistical analyses were performed using SigmaPlot (Version 13.0, Systat Software, Inc., San Jose, USA). All reported tests passed the Shapiro-Wilk normality test. All reported P-values are two tailed and are the result of paired t-tests, unless otherwise stated.

RESULTS AND DISCUSSION

Raw Hand-Rinse

Estimated total staphylococci CFU counts on MSA (TS-MSA) before and after UV-C exposure (220 $mJ.cm^{-2}$) from the five participants made up the majority of the total TSA CFU counts (T-TSA) (90 % & 75 % respectively). More importantly, the $\log_{10}$ of the TS-MSA to T-TSA ratio (displayed as a percentage) of before and after UV-C exposure was 99 % and 97 % respectively. A paired t-test (see Table 4.1) was performed comparing the $\log_{10}$ reduction from UV-C exposure within the T-TSA and TS-MSA assays from the five participants; the $\log_{10}$ reductions of T-TSA and TS-MSA were not found to be significantly different ($P = 0.112$). The similarity in $\log_{10}$ reduction of the T-TSA and TS-MSA assays (means of 2.1 and 2.2, respectively) suggests that TS-MSA was representative of bacterial reduction by UV-C irradiation of the hand-rinse water from the five participants. Figure 4.1a depicts the T-TSA and TS-MSA assays before and after exposure to UV-C. The T-TSA and TS-MSA concentrations reported in this study are not considered representative of concentrations across all wash-basin greywater sources, as soap was not used and only 40 mL was used to wash participants' hands; rather, the significance of this study is exhibited in the consistently similar T-TSA and TS-MSA concentrations, implying the vast majority of recovered bacteria were staphylococci.

Although the results from the five participants were consistent (See Table B.1), more replication of this study on a variety of greywater sources is necessary to confidently suggest total staphylococci as a performance surrogate for greywater treatment. A study by Abshire and Dunton (1981) showed that *S. aureus* was more resistant to UV-C than the saprozoic pathogen *Pseudomonas aeruginosa* (also typically present in greywater (Gilboa and Friedler 2007)); however, a study by Gilboa and Friedler (2007) reported *S. aureus* to be the least resistant to UV-C irradiation when compared to faecal coliforms (FC), *P. aeruginosa*, and *Clostridium perfringens* at low doses. However, as shown in this study, given the dominating concentrations of *Staphylococcus* spp. in raw greywater (Benami *et al.* 2016, Zimmerman *et al.* 2014), *Staphylococcus* spp. may still be a practical representative surrogate for total bacterial pathogenic reduction in greywater.

Although TS-MSA represented the majority of T-TSA, a paired t-test showed that when T-TSA and TS-MSA assays were compared (both before and after exposure) the $\log_{10}$ means were statistically significantly different (P = 0.04 for both) (see Table 4.1). However, when assessing MSA and TSA assays (both before and after exposure) prior to Lysostaphin™ confirmations, no statistical difference was observed (P = 0.78 & 0.73 respectively). S*taphylococcus* spp., some *Micrococcus* spp., and some *Enterococcus* spp. are of the few organisms known to be capable of growing in the high salt environment in MSA (Quiloan *et al.* 2012, Rodríguez *et al.* 1994); it is likely that the majority of the Lysostaphin™ negative MSA CFU were *Micrococcus* spp., as micrococci are one of the more abundant bacteria genera inhabiting human skin (Kloos and Musselwhite 1975), however no further confirmations were performed to determine what the non-*Staphylococcus* spp. were. Figure 4.1a displays the T-TSA and TS-MSA assays before and after UV exposure.

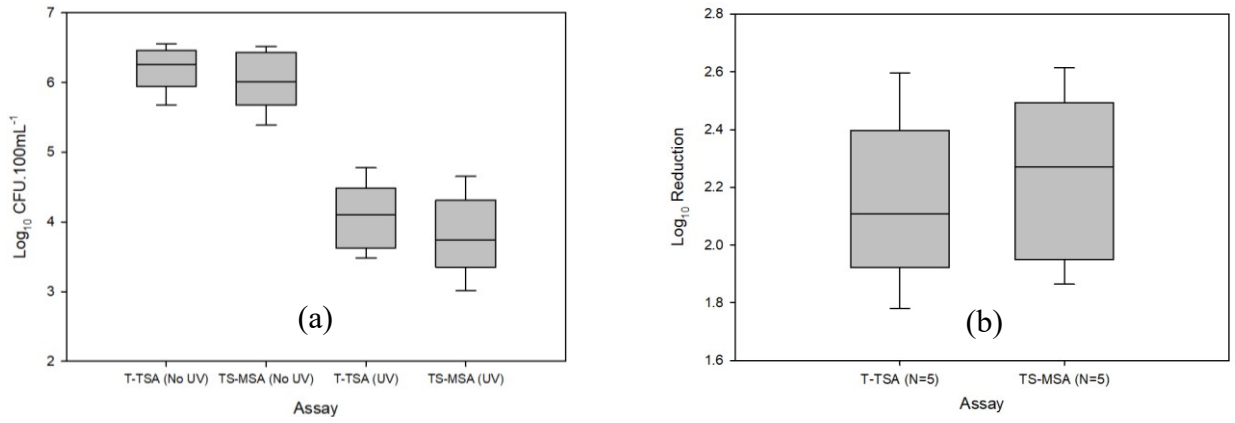

Figure 4.1: Hand-rinse bacteria exposed to 256 nm UV-C irradiation: (**a**) Log_{10} CFU.100mL^{-1} on TSA (T-TSA) and total staphylococci on MSA (TS-MSA) before and after exposure to dose of 220 mJ.cm^{-2} (N = 5); (**b**) Log_{10} reduction of T-TSA and TS-MSA (N = 5).

Table 4.1: Log_{10} colony forming units per 100 mL of raw hand-rinse water before and after exposure

Assay	**Log_{10} CFU.100mL^{-1} ± (SD)**		
	0 mJ.cm^{-2}	**220 mJ.cm^{-2}**	**Reduction**
T-TSA	6.2 ± (0.3)	4.1 ± (0.5)	2.1 ± (0.3)
TS-MSA	6.0 ± (0.4)	3.8 ± (0.6)	2.2 ± (0.3)
Paired t-test	0.04[a]	0.04[a]	0.112[a]

[a] Two-tailed P-value comparing means.

A notable concern within this study was the lack of bacterial reduction by 256 UV-C at a dose of 220 mJ.cm^{-2}, which should have yielded at least a 5-log_{10} reduction in viable bacteria, as previously shown by Shoults and Ashbolt (2017a). This is not surprising, however, as Winward *et al.* (2008) showed a significant decrease in UV-C efficacy of bacterial reduction in water with high turbidity and low UV transmittance. The 256 nm absorbance (A_{256} nm) readings ranging from 0.164 to 0.360 (see Table B.1) and the applied dose was not normalized for turbidity or absorbance, as given in the unadjusted Equation 4.1; the A_{256} readings would likely have been increased by the use of soap in this study, and thus soap wasn't used. Two future considerations should be given towards future studies involving UV-C irradiation of raw hand-rinse greywater:

1. Pre-treatment prior to UV-C irradiation is necessary in order to achieve more than a 2-$\log_{10}$ reduction of endogenous bacteria due to particulate shielding and organic quenching effects; and
2. Equation 4.1 describing the delivered UV-C intensity requires adjustment to account for shielding/quenching from high turbidity/organics in greywater samples.

A study by Liu and Zhang (2006) examined the effects of turbidity on the efficacy of bacterial and coliphage reduction by UV; although there was not a major difference in UV-C efficacy between turbidities of 0.5 and 4 Nephelometric Turbidity Units (NTU), there was a significant difference between 4 and 12 NTU. This suggests pre-treatment removal of particles to a turbidity of < 12 NTU is probably necessary for optimal UV-C efficacy.

Hand-Rinse Isolates

The aim of this portion of the study was to examine the variability of resilience of environmental staphylococci isolates. The following *Staphylococcus* spp. were isolated from the five participants' hands (all multiples of species are from different participants): *S. aureus* (1), *S. capitis* (2), *S. epidermidis* (3), *S. haemolyticus* (2), *S. hominis* spp. *hominis* (*S. hominis*) (1), *S. pasteuri* (3), and *S. warneri* (2). The effects of 256 nm UV-C at a dose of 11 $mJ.cm^{-2}$ were assessed in order to determine the variance of resistance to UV within and between *Staphylococcus* species; the results are displayed in Table 4.2:

Table 4.2: Log_{10} reduction of ATCC and wild *Staphylococcu*s isolates

Bacteria	**Log_{10} reduction at 11.0 mJ.cm^{-2} ± (SD)**
S. aureus (ATCC 25923)	4.9 ± (0.0)
S. aureus (i)	> 5.2
S. capitis (ii)	> 5.7
S. capitis (iii)	> 6.3
S. lentus (unknown)	5.0 ± (0.0)
S. epidermidis (ii)	> 5.7
S. epidermidis (iiia)	> 6.1
S. epidermidis (iiib)	> 5.6
S. haemolyticus (iv)	3.4 ± (0.1)
S. haemolyticus (v)	4.4 ± (0.1)
S. hominis (v)	1.6 ± (0.1)
S. pasteuri (iii)	> 5.8
S. pasteuri (iv)	> 5.5
S. pasteuri (v)	5.1 ± (0.1)
S. warneri (i)	> 5.7
S. warneri (ii)	> 5.5

Note: i-v refers to the five participants from whom the greywater isolates were derived.

When exposed to 256 nm UV-C at a dose of 11 mJ.cm^{-2}, the majority of isolates experienced at least a 5-log_{10} reduction. However, there was significant variance in log_{10} reductions after UV exposure between different *Staphylococcus* spp., with a less significant variance within each species. *S. hominis* and *S. haemolyticus* were significantly more resistant to UV-C irradiation than the other isolates. The full decay equations for *S. hominis* and *S. haemolyticus* were $y = 0.23x - 0.96$ ($R^2 = 0.97$) and $y = 0.30x + 0.25$ ($R^2 = 0.91$), respectively, where (y) is log_{10} reduction, and (x) is the dose in mJ.cm^{-2}. Decay equations were not obtained for the other bacteria, due to insufficient plate counts at each exposure assay. A previous 2017 study by Shoults & Ashbolt determined that *Staphylococcus* spp. (as represented by *S. aureus* and *S. epidermidis* [ATCC 12228 as used in Shoults and Ashbolt (2017a) was confirmed by MALDI-MS VITEK™ verification, but characterized as *S. lentus* by VITEK2™ phenotyping]) was more sensitive to UV-C irradiation than some FIB (Shoults and Ashbolt 2017a). However, the relative resistance of *S. hominis* and *S. haemolyticus* suggests *Staphylococcus* spp. may be as resistant to

UV as FIB. However, further study is required to understand the full range of resistance within *Staphylococcus* spp.

Effects of UV Blocker Oxybenzone on UV-C Efficacy

Though UV blockers such as oxybenzone are known to quench the effects of UV when used on skin (common compound in sunscreens), the direct impacts of sunscreens and other personal care products (PCP) on the efficacy of UV irradiation for domestic water treatment are not well known. Figure 4.2 illustrates the effects of oxybenzone on the efficacy of reduction of *S. aureus* (ATCC 29523) by 256 nm UV-C.

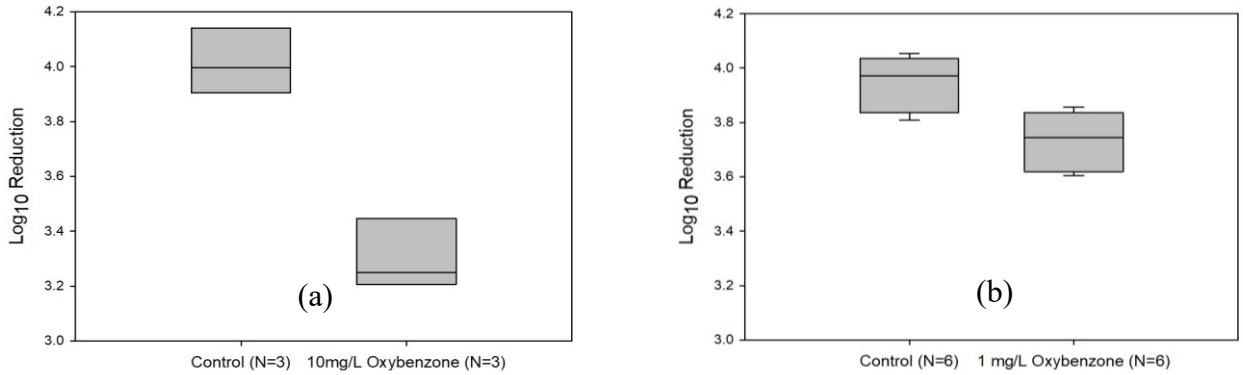

Figure 4.2: UV-blocking of oxybenzone when irradiating suspended *S. aureus* at 11.8 mJ.cm^{-2}: (a) 10 mg.L^{-1} oxybenzone; (b) 1 mg.L^{-1} oxybenzone.

Samples containing oxybenzone (concentrations of 10 mg.L^{-1} and 1 mg.L^{-1}) exhibited significantly lower $\log_{10}$ reductions ($P = 0.003$ & 0.01, respectively) than their respective negative controls (containing no oxybenzone). This may help explain the lack of bacterial reduction of raw hand-rinse greywater (see Figure 4.1) when exposed to a dose of 220 mJ.cm^{-2}; it is expected many of the hand-rinse samples contained PCP's, creams, or sunscreen agents. Although it is generally well understood that pre-treatment is necessary prior to UV irradiation, it is important to know which compounds are to be removed and to what extent. PCP's can be difficult to remove from greywater (Christian 2007, Kasprzyk-Hordern *et al.* 2009); however, depending on the intended end-use of the treated effluent, removal may be necessary if UV is to be the sole disinfection step.

Summary and Conclusions

The research displayed in this paper evaluated the efficacy of 256 nm UV-C in treating raw hand-rinse water, analyzed the efficacy of total culturable *Staphylococcus* spp. as an endogenous

surrogate group to represent bacterial reduction, examined the variability of wild *Staphylococcus* spp. isolates when exposed to UV-C, and assessed the impacts of suspended oxybenzone on the efficacy of UV-C irradiation. It is clear pre-treatment is necessary prior to UV irradiation for a $\log_{10}$ reduction of greater than two to be observed; the presence of PCP (such as oxybenzone) as well as turbidity can have a negative effect on the efficacy of UV-C irradiation. Given the high prevalence of *Staphylococcus* spp. found in the five samples of raw hand-rinse water and more generally reported on human skin, future studies should investigate the surrogate use of endogenous *Staphylococcus* spp. for bacterial reduction in greywater reuse systems to better understand any limitations at field-scale. Further research should be performed to assess the $\log_{10}$ reductions by other disinfection methods of *Staphylococcus* spp. relative to other pathogens of concern to determine if *Staphylococcus* spp. would be an adequate endogenous surrogate for other disinfection processes. Finally, the results in this study have shown that the sunscreen oxybenzone can have a negative effect on the efficacy of UV irradiation; further research should also address the role of other PCPs on the efficacy of UV irradiation.

Supplementary materials: The following are available online at www.mdpi.com/link, Table B.1: Raw hand-rinse water reduction, Figure B.1: MS2 Literature Comparison (Liu and Zhang 2006).

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Author contributions: David Shoults and Nicholas Ashbolt conceived and designed the experiments; David Shoults performed the experiments, analyzed the data, and wrote the paper as part of his M.Sc. study under the supervision of Nicholas Ashbolt.

Conflicts of interest: The authors declare no conflict of interest.

CHAPTER 5: DECREASED EFFICACY OF UV INACTIVATION OF *STAPHYLOCOCCUS AUREUS* AFTER CYCLIC EXPOSURE AND REGROWTH: CONSIDERATIONS FOR GREYWATER REUSE SYSTEMS

As submitted to the *International Journal of Hygiene & Environmental Health*

ABSTRACT

UV disinfection is a relatively simple and cost-efficient disinfection method, especially for in-home greywater treatment. In this study, a bench scale experiment was performed using a collimated 256nm UV-C beam to determine if UV-resistant pathogenic bacteria such as *Staphylococcus aureus* may become enriched in a semi-recirculating greywater system with UV as the sole disinfection step. A statistically significant ($P < 0.001$) decreasing trend in UV-C efficacy was observed between the 1st and 6th growth-exposure cycles of *S. aureus* (ATCC 25923), resulting in a 1.5 decrease in $\log_{10}$ removal ($P < 0.00000$) by the 5th iteration. An eleven-point dose-response curve of the 7th iteration of *S. aureus* was enumerated and compared to the dose-response curve of the original strain; due to a longer shoulder period and a decay constant of lesser degree, the dose required for a 4-log reduction of the enriched *S. aureus* was estimated to be ~1.9 times greater (22.0 mJ.cm^{-2} versus 11.8 mJ.cm^{-2}). This suggests *S. aureus* (and possibly other opportunistic pathogens) may become enriched within a greywater system with UV-C irradiation as the sole disinfection step.

INTRODUCTION

Increasing population growth in relatively water-scarce regions along with an increase in personal water consumption have greatly contributed to the urban water deficit faced around the world (Schiermeier 2014). The concept of reusing greywater is becoming more popular; greywater, domestic household wastewater without input from the toilet, is a valuable commodity, which can be utilized to reduce domestic potable water usage. Here the focus is on utilization of domestic greywater, as one option to provide more sustainable water services (Schoen *et al.* 2014). Typical greywater treatment systems may involve some form of pre-treatment with filtration, then secondary/tertiary treatment by chemical (*e.g.* chlorine disinfection) (Al-Gheethi *et al.* 2015), biological (*e.g.* membrane bioreactor) (Atasoy *et al.* 2007), or physical processes (*e.g.* ultra violet irradiation) (Friedler and Gilboa 2010). This study focuses on some of the considerations of ultra violet (UV) irradiation as a disinfection process for greywater treatment. As with most disinfection technologies, UV has its disadvantages; in particular, when bacteria are stressed they have the potential to adapt resistance to the stressor(s) (Friedler *et al.* 2011, Gayán *et al.* 2014, Jiang *et al.* 2016). Certain bacteria exhibit various mechanisms, such as light and dark repair mechanisms for repairing damaged cell membranes

and cellular components (Masschelein 2002, Nebot Sanz *et al.* 2007). Additionally, damage to cell membrane (Pigeot-Rémy *et al.* 2012) and therefore adaptation may involve vertical and subsequent horizontal gene transfer from resistant bacteria and/or adaptation by upregulation of UV quenching molecules from within an existing genome, also given the intrinsic resistance seen in other Gram-positive bacteria (Davies-Colley *et al.* 2007, Williams *et al.* 2007). Hence with greywater reuse, especially in a semi-continuous loop system, adaptation is always possible, such as DNA repair following damage caused by UV (Friedler *et al.* 2011). Adaptation is problematic, as pathogens may become resistant to the disinfection method greater than the surrogates used to measure treatment performance, hence leading to an unrecognized increase in public health risk.

Each specific wastewater presents different contaminants, both chemical and biological, which require varying levels of reduction. Greywater presents particular challenges for treatment; unlike municipal wastewater, enteric pathogens are not the only organisms of concern (Zimmerman *et al.* 2014); skin bacteria such as staphylococci are documented as a consistently prevalent genus present in greywater (Gross *et al.* 2007, Linden *et al.* 2012, Zimmerman *et al.* 2014). *Staphylococcus aureus*, which colonizes some 30 % of humans (Plano *et al.* 2011) is a potential pathogenic *Staphylococcus* species capable of causing systemic infections including bacteremia, pneumonia, endocarditis, and osteomyelitis (Lowy 1998). Most staphylococci grow on human skin; this makes the treatment and reuse of household greywater potentially problematic as skin-pathogenic bacteria may survive disinfection and return to the host (humans), where they may cause infection. Hence, the aim of this work was to investigate if a recirculating greywater system with only UV disinfection may facilitate regrowth of UV resistant *S. aureus* that may be pathogenic or represent a model for other skin pathogens.

MATERIALS AND METHODS

Microbiological Components

Two *Staphylococcus* strains obtained from the American Type Culture Collection (ATCC) and two wild *Staphylococcus* isolates obtained from a hand-rinse sample from one of the authors were used as test specimen. *S. aureus* (ATCC 25923) and *Staphylococcus epidermidis* (ATCC 12228 as used in Shoults and Ashbolt (2017a) was confirmed by MALDI-MS VITEK^{TM}, characterized as *Staphylococcus lentus* upon VITEK2^{TM} phenotyping) were selected as test organisms for this experiment based on their high skin prevalence on humans (Coates *et al.*

2014) and relatively high reporting of *S. aureus* and total staphylococci concentrations in raw greywater (Gross *et al.* 2007, Linden *et al.* 2012, Zimmerman *et al.* 2014). A VITEK™ (2 COMPACT) was used to verify the genera and species of two suspected hand-rinse sample staphylococci isolates, which were confirmed to be *S. aureus* and *Staphylococcus warneri*. MS2 bacteriophage (ATCC 15597-B1) was used as a control specimen with *Escherichia coli* ATCC 15597 as host cell.

Experimental Setup

Overnight cultures of test specimens were grown in tryptic soy broth (TSA). Once incubated, the cell broths were diluted to 1:100 in sterile deionized (DI) water within a sterile 60 mm Petri plate containing a sterile 5 mm x 2 mm stir bar and magnetic mixer operating at 400 rpm to facilitate mixing without a vortex forming. DI water was used in order to minimize potential contact with residual chlorine, which could have an inactivation effect on the cells (Zyara *et al.* 2016). A control using sterile DI water was performed to investigate any loss in viability due to osmotic pressure change. However, no significant difference in $\log_{10}$ CFU count was observed from 100 seconds after pipetting from the TSA liquid medium (the time needed to dilute the cells to a countable dilution) to 20 min (the estimated maximum time cells might be suspended in DI water throughout the experiment) ($P = 0.286$ & 0.289; for *S. aureus* and *S. lentus*, respectively). An AquaSense Pearl Beam collimated LED UV reactor (Florence, KY USA) with a peak wavelength of 256 nm and a half bandwidth of 11.5 nm was used to deliver 256 nm UV-C irradiation to *S. aureus* and *S. lentus* suspended in water using an adapted EPA protocol (USEPA 2006). Equation 5.1 was used to calculate the effective intensity (E_{ave}) of the collimated beam based on measurable variables(NSF International 2014):

$$E_{ave} = 0.98\left[\frac{E_0}{L}\left(\frac{(1-A)^L-1}{ln(1-A)}\right)\right] \qquad \text{Equation 5.1}$$

The incident intensity (E_0) was measured using a NSF certified radiometer (UVP Radiometer, Model UVX-25). The water height (L) was measured to 1 cm (28.3 mL in a 60 mm cylindrical Petri dish), and a spectrophometer (Thermo Scientific Genesys 10S UV-VIS) was used to measure the absorbance (A_{256} nm) in the solution. The resulting E_{ave} was then multiplied by the exposure time (seconds) in order to calculate the resulting dosage in $mJ.cm^{-2}$.

Prior to irradiating the sample, 100 µL of the sample was plated on tryptic soy agar (TSA) plates in triplicate at the appropriate dilution to effectively measure colony forming units (CFU) of the control. An estimated dose for a 4-$\log_{10}$ reduction of *S. aureus* of 11.8 mJ.cm^{-2} was used as a starting point to attempt to isolate *S. aureus* cells that may be more resistance to UV (Shoults and Ashbolt 2017a). The collimated beam was placed over the Petri dish containing the 1:100 DI suspension of *S. aureus* which was then irradiated to the specified dose; samples were diluted appropriately and 100µL was plated on TSA plates in triplicate to assay the remaining colony forming units (CFU); with the $\log_{10}$ reduction based on the CFU/100 µL count of the control at 0 mJ.cm^{-2} according to Equation 5.2:

$$\text{Log}_{10}\, Reduction = Log\left(\frac{\frac{CFU\ Control}{100mL}}{\frac{CFU\ Dosage}{100mL}}\right) \qquad \text{Equation 5.2}$$

Regrowth of Irradiated Cells

A 200 µL aliquot of the irradiated sample was transferred to 5 mL TSB and incubated for 24 h to allow for regrowth of the surviving cells. The experiment was then repeated on a suspension containing the regrown *S. aureus*. This experimental sequence was performed as many as six times per specimen. A consistent dose was used for each growth-irradiation cycle to determine if there was a decreasing trend in $\log_{10}$ reductions. *S. aureus* (ATCC 25923) and *S. lentus* (ATCC 12228) were exposed to 11.8 mJ.cm^{-2} and 17.0 mJ.cm^{-2}, respectively, which are the estimated dosages for a 4-$\log_{10}$ reduction of each (Shoults and Ashbolt 2017a). *S. aureus* (wild) and *S. warneri* (wild) were both exposed to subsequent doses of 15, 30, and 40 mJ.cm^{-2}; these doses were used to explore the potential effects of bacterial selection by UV doses that might be used in a treatment system, with 40 mJ.cm^{-2} and 16 mJ.cm^{-2} being the minimum UV doses for Class A and Class B UV treatment systems, respectively (NSF International 2014).

Dose-Response Curve

An 11-point test was performed on the 7th iteration of *S. aureus* (ATCC 25923) (denoted as *S. aureus*^{7th} from this point forth) in accordance with NSF's ultraviolet microbiological water treatment systems document (NSF International 2014). Relative doses (0 %, 15 %, 30 %, 45 %, 60 %, 75 %, 90 %, 105 %, 120 %, 135 %, and 150 %) based on the estimated dose required for a 4-log reduction (e.g. 15 % of 11.8 mJ.cm^{-2} = 1.77 mJ.cm^{-2}) were assayed to determine a dose-response curve for *S. aureus*^{7th} (Equation 5.2).

Control Experiments

Two additional control experiments were performed to ensure homogeneity with literature as well as consistency throughout the experiments. An 11-point dose-response curve using MS2 bacteriophage was performed using the above protocol and assayed appropriately (EPA 2001); this curve was then compared to a UV-C study performed by Liu and Zhang (2006) using MS2 and was found to be within the same $\log_{10}$ reduction range (see Figure B.1 for graph comparison). To ensure absorbance did not change between experiments and confound the results, a one-way ANOVA analysis was performed; the hypothesis that the average irradiances (using Equation 5.1 as a function of absorbance) differed between days was rejected ($P = 0.629$).

Methodical Exceptions

The procedure for *S. aureus* (wild) and *S. warneri* (wild) had the following difference: overnight TSB cultures were centrifuged to form pellets which were re-suspended in a sterile 0.85 % NaCl buffer, vortexed, then pipetted into a petri dish containing NaCl buffer for UV irradiation. The rational for making this procedural adjustment was to remove the TSB to have a higher concentration of suspended cells without the presence of TSB increasing the turbidity.

Statistical Analyses

SigmaPlot (Version 13.0, Systat Software, Inc., San Jose, CA) was used for all statistical analysis. Paired t-tests were used for comparing mean data, and two-tailed p-values are reported. Linear regression analysis was used to analyze the presence of potential resistance trends. All reported statistics passed Shapiro-Wilk normality test.

RESULTS AND DISCUSSION

The majority of studies investigating the effects of repeated UV exposure cycles have used *E. coli*, which report some degree of decreased UV efficacy over the course of several cycles of UV exposure and regrowth (Alcántara-Díaz *et al.* 2004, Ewing 1995, Wright and Hill 1968). No study was identified that examined the effects of multiple exposures of UV-C towards Gram-positive wastewater bacteria (such as staphylococci or enterococci), however, data exists for increased resistance following prolonged sunlight exposure (Hartke *et al.* 1998). Gram-positive cells tend to be more intrinsically resistant to UV stress (Gehr *et al.* 2003, Williams *et al.* 2007); potential increases in resistance may be problematic for treatment systems using UV irradiation. Previous UV resistance studies have examined in partial, the adaptive mechanisms present in

enriched cells (Alcántara-Díaz *et al.* 2004), however this study did not attempt to identify such mechanisms, rather to determine if adaptive resistant trends can occur in *Staphylococcus* spp., and therefore assess the relevance to greywater reuse.

Iterative UV-C Inactivation of ATCC Strains *S. aureus* and *S. lentus*

The results of UV irradiation delivered to *S. aureus* at a dose of 11.8 $mJ.cm^{-2}$ over six exposure-regrowth cycles are displayed in Figure 5.1a. A regression analysis indicated a declining trend in log_{10} reduction ($P < 0.001$). While it is difficult to say what the mechanism(s) causing *S. aureus* resistant may be, it is important to note that Gayán *et al.* (2014) reported intraspecific variation in UV resistance amongst *S. aureus* strains of 1.3 fold when exposed to UV-C at 254 nm. Given the single strain (ATCC 25923) used in the current study, however, there appears to be a significant trend of increasing UV resistance within the surviving population. The higher variability of the second and third exposure iteration shown in Figure 5.1a can be explained by the two separate runs of the experiment. The first experimental trial yielded a significant difference in log_{10} reduction between the first and second exposure iteration; however, in the second trial a similar change in reduction was not observed until the third iteration, resulting in the large variation in the combined data in Figure 5.1a. The variation in results between the two trials suggests a randomization effect may play a role in the adaptation of cells. The results of UV irradiation delivered to *S. lentus* at a dose of 17.0 $mJ.cm^{-2}$ are displayed in Figure 5.1b. A regression analysis rejected the hypothesis that a declining trend in log_{10} reduction existed over the five iterations of the experiment ($P = 0.315$). While it cannot be concluded that *S. lentus* lacks the adaptive mechanisms that *S. aureus* appears to exhibit, *S. aureus* appeared to be more readily enrichment for UV-resistance.

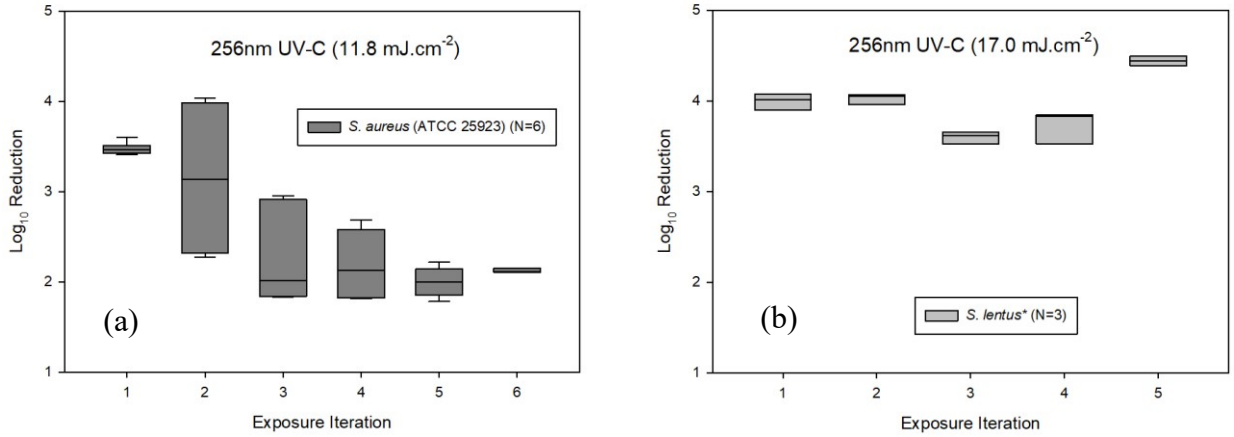

Figure 5.1: Cyclic growth-irradiation of bacteria using 256nm UV-C (**a**) *S. aureus* (ATCC 25923) at 11.8 mJ.cm^{-2} (N=6); (**b**) *S. lentus* at 17.0 mJ.cm^{-2} (N=3). *ATCC 12228 as used in Shoults and Ashbolt (2017a) was confirmed by MALDI-MS VITEK^{TM}, characterized as *Staphylococcus lentus* upon VITEK2^{TM} phenotyping.

Iterative UV-C Inactivation of Wild *S. aureus* and *S. warneri* Isolates

The two wild staphylococci, *S. aureus* and *S. warneri* were examined to determine if the resistance trend exhibited in Figure 5.1a could be replicated with wild isolates. As shown in Figure 5.2, neither the wild *S. aureus* nor *S. warneri* exhibited a resistance trend after four growth-exposure cycles when exposed to 15, 30, or 40 mJ.cm^{-2}. Caution should be taken when analyzing the data shown in this study; there are potential explanations for the lack of decrease in UV efficacy. Certain bacteria may require more generations of reproduction than others to exhibit environmental adaptation; hence more growth-exposure cycles may be necessary to show a significant change in UV resistance (Alcántara-Díaz *et al.* 2004).

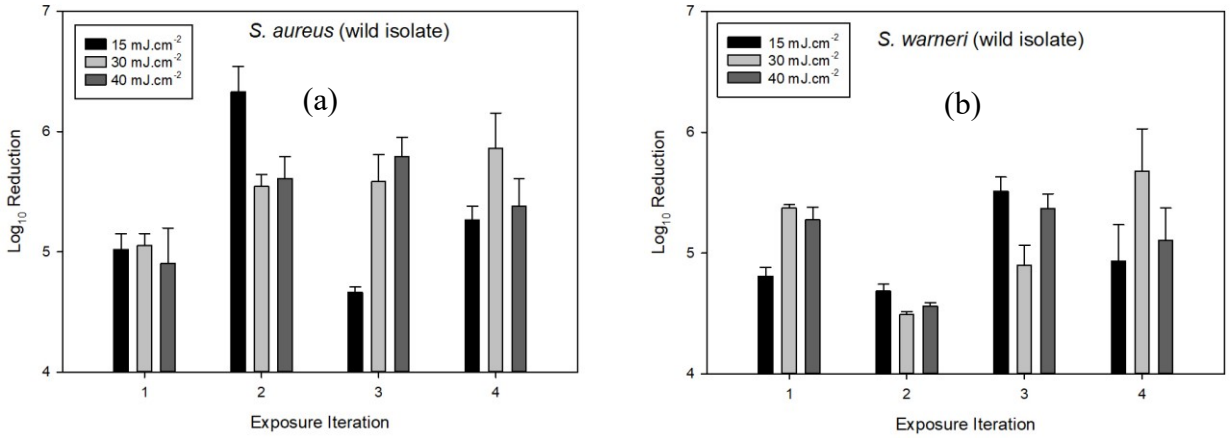

Figure 5.2: Cyclic growth-irradiation of bacteria at 256nm UV-C doses of 15, 30, and 40 mJ.cm^{-2} (**a**) *S. aureus* (wild isolate) (N=3); (**b**) *S. warneri* (wild isolate).

UV Dose-Response Curve for *S. aureus*

An 11-point dose-response curve was generated for progeny from the 7th iteration of *S. aureus* (ATCC 25923) (*S. aureus*^{7th}) and was compared to the 11-point dose response curve of the parental *S. aureus* ATCC 25923 strain (to be referred as *S. aureus*^{1st}) generated in a previous study by Shoults and Ashbolt (2017a); the estimated dose required for a 4-log reduction of *S. aureus*^{7th} was 22.0 mJ.cm^{-2} which is ~1.9 times higher than the dose required for a 4-log$_{10}$ reduction of *S. aureus*^{1st} (11.8 mJ.cm^{-2}). The first order decay coefficients (k) of the linear portions of the UV dose-response curves for *S. aureus*^{1st} and *S. aureus*^{7th} depicted in Figure 5.3 were estimated to be k_{1st} = -0.45 (R^2 = 0.990) and k_{7th} = -0.24 (R^2 = 0.997), respectively. *S. aureus*^{7th} had a longer shoulder period, as well as a lower k-value. Firstly, the shoulder effect can likely be attributed to the action of DNA repair mechanisms, requiring multiple hits on a single organism for cell death (Gayán *et al.* 2014). Secondly, the lower magnitude of the k-value of *S. aureus*^{7th} suggests it is intrinsically more resistant to UV-C (256nm) irradiation than *S. aureus*^{1st}.

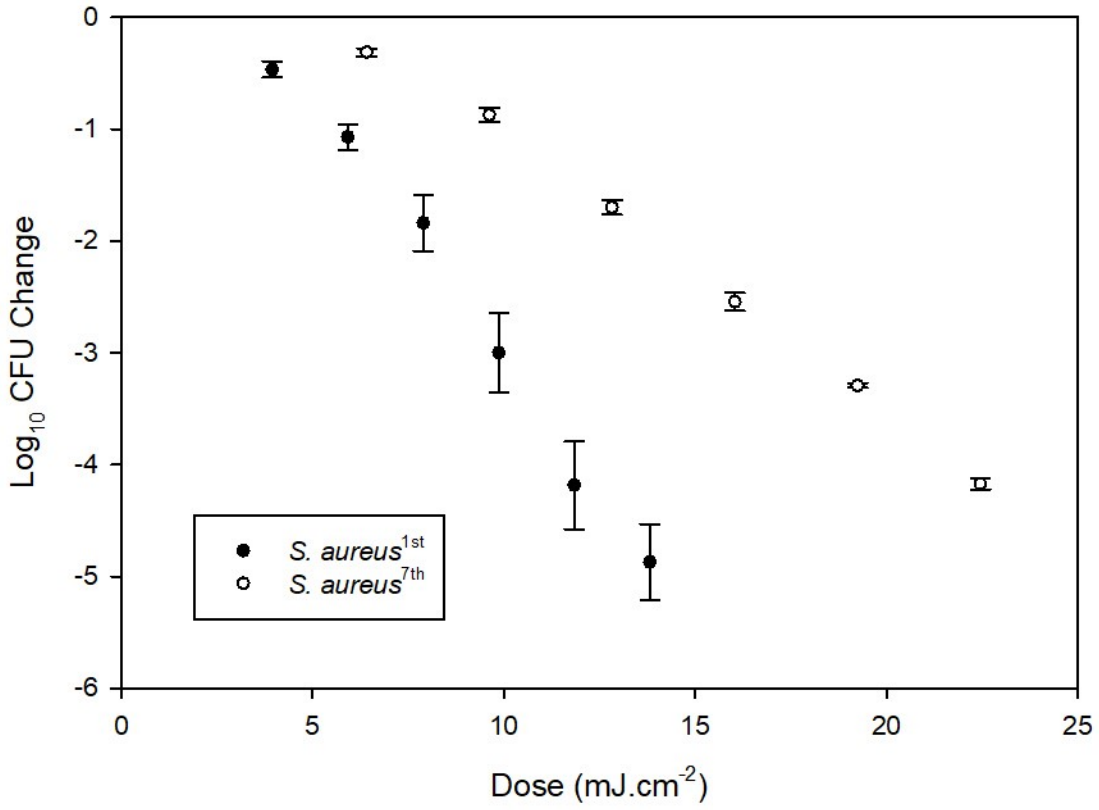

Figure 5.3: 11-point dose response curves of *S. aureus*[1st] and *S. aureus*[7th] when exposed to 256nm UV irradiation

Total Staphylococci as UV-Treatment Surrogate for Greywater

As we have previously discussed (Shoults and Ashbolt 2017a), total staphylococci provide several useful attributes as endogenous treatment surrogates for greywater performance testing. Although the results indicated total staphylococci was more susceptive to UV-C irradiation than other traditional faecal indicator microorganisms, they may still serve as a suitable surrogate due to their consistently high concentrations relative to traditional faecal indicator bacteria (FIB) in greywaters (Casanova *et al.* 2001, Gross *et al.* 2007, Linden *et al.* 2012, Shoults and Ashbolt 2017a, Zimmerman *et al.* 2014). In addition, the data analyzed in the current work shows that one important pathogenic member, *S. aureus* may be a more conservative surrogate for representing pathogenic risk than previously thought, due to the potential for enrichment in a recirculating UV disinfection system. When compared to the dose required for a 4-$\log_{10}$ reduction of *S. aureus*[1st] (11.8 mJ.cm^{-2}), the dose required for a 4-$\log_{10}$ reduction of *S. aureus*[7th] (21.8 mJ.cm^{-2}) was more similar to the doses required for a 4-$\log_{10}$ reduction of *E. coli*

(ATCC 13115), *Enterococcus faecalis* (ATCC 29212), and *Enterococcus casseliflavus* (ATCC 9199), which were 20.4, 25.6, and 22.1 $mJ.cm^{-2}$ respectively (Shoults and Ashbolt 2017a). In addition to total staphylococci being reported as 3 to 5-log_{10} higher in abundance than traditional FIB in untreated laundry greywater (Zimmerman *et al.* 2014), the potential of increased resistance of *S. aureus* as described by the current study may cause total staphylococci to be a more conservative surrogate for treatment performance than previously thought. However, this increased resistance may be confounded by the potential selection of other enteric bacteria with some capability of regrowth within the greywater system (Friedler and Gilboa 2010, Jahne *et al.* 2016) which have been consistently shown to be able to adapt over several growth-UV exposure cycles (Alcántara-Díaz *et al.* 2004, Ewing 1995, Wright and Hill 1968).

FUTURE RESEARCH

Given *S. aureus* can theoretically become enriched in a circulating system with UV as the disinfection process, further research is needed to determine the scale to which the results shown in this study may apply in practice. Without running a wet study examining greywater from recirculating greywater systems over the course of several weeks, it is difficult to determine whether or not the trend exhibited in Figure 5.1a would be found in a real greywater system. Additionally, to better understand the effects of staphylococci as a surrogate for treatment performance in a greywater reuse system, we must better understand the levels of adapted bacteria (both staphylococci and other bacteria such as *E. coli*) being reintroduced into the cycle.

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CHAPTER 6: DISCUSSION

It is important that Chapters 3, 4, and 5 be considered together. While Chapter 3 estimated the UV kinetics of total staphylococci by averaging the kinetics of *S. aureus* and *S. epidermidis*, the results discussed in Chapter 4 (Table 4.2) need to be considered, which showed *S. hominis* subsp. *hominis* and *S. haemolyticus* were considerably more resistant to UV irradiation than the other *Staphylococcus* species (*S. aureus*, *S. capitis*, *S. epidermidis*, *S. lentus*, *S. pasteuri*, and *S. warneri*). It is also important to estimate the UV kinetics of total staphylococci as a $\log_{10}$ normalized function based on relative abundance and individual decay kinetics. A study by Kawamura *et al.* (1998) reported relative abundances of six of the eight *Staphylococcus* species identified in Chapter 4; *S. epidermidis* (31.3 %), *S. aureus* (23.3 %), *S. haemolyticus* (12.2 %), *S. hominis* (4.0 %), *S. capitis* (3.9 %), *S. warneri* (2.2 %), and other *Staphylococcus* spp. (23.1 %). A study by Kloos (1980) described *S. epidermidis* and *S. hominis* as the most prevalent and persistent *Staphylococcus* spp. on human skin, comprising more than 65 % of the staphylococci isolated on normal glabrous skin (accounting for the majority of the skin area). Given the relatively high abundance of *S. hominis* on human skin, the persistence of *Staphylococcus* spp. during UV inactivation would likely be higher than estimated in Chapter 3.

While it is difficult to conclusively understand the difference in UV decay kinetics between the aforementioned *Staphylococcus* spp., it is hypothesized here that the cell structure of *S. hominis* played a major role in the relative resistance to UV. A study by Kloos and Schleifer (1975) characterized *S. aureus*, *S. haemolyticus*, *S. warneri*, and *S. epidermidis* as having the majority of cell arrangements as pairs and singles; *S. capitis* was generally mixed between primarily pairs/singles and tetrads; *S. hominis* was the only *Staphylococcus* spp. characterized as having majority tetrad cell structure. If cell structure plays a role in the relative UV resistance of certain bacteria, this may be a confounding factor in comparing data from collimated UV irradiance of inoculated bacteria to UV irradiance of indigenous bacteria. Some protocols may require vortexing of bacteria inoculum prior to irradiation in order to reduce clumping of bacteria; this may confound results when compared to naturally clumped indigenous bacteria groups potentially found in greywater.

The research performed in this thesis has shown that total staphylococci may provide an adequate measure of total bacterial reduction within a greywater treatment disinfection step. Of equal importance is total staphylococci's potential measure as an index organism for the potential pathogen *S. aureus*. The study by Zimmerman *et al.* (2014) showed a significant correlation between the presence of total staphylococci and *S. aureus*; this is no surprise as *S. aureus* has been shown to colonize 30 to 40 % of humans (Kluytmans *et al.* 1997), being a member of the *Staphylococcus* genus. Figure 3.1 and Table 4.2 showed that *S. aureus* was less resistant to UV irradiation than other *Staphylococcus* spp.; given the relative UV susceptibility of *S. aureus*, the reduction of total staphylococci may provide a conservative measure for the reduction of *S. aureus* by UV irradiation. Although the results shown in Chapter 5 showed *S. aureus* may theoretically increase in resistance and become enriched, the $\log_{10}$ reductions of total staphylococci would reflect this change in resistance, and therefore the kinetics between total staphylococci and *S. aureus* would still be consistent, however somewhat less conservatively. With an estimated median effective dose (ED_{50}) of approximately 10^3 *S. aureus* organisms per cm^2 (Singh *et al.* 1971), and *S. aureus* concentrations in greywater measured as high as 10^7 CFU.100 mL^{-2} (Burrows *et al.* 1991), it is conceivable that upwards of 5-$\log_{10}$ reductions of *S. aureus* are needed for safe non-potable reuse of greywater.

CHAPTER 7: CONCLUSIONS AND RECOMMENDATIONS

There is a collective need for guidelines to be developed for consistent analysis of the microbial composition of greywater; the inconstant findings of the literature review displayed in Chapter 1 reinforce this need and to extend beyond FIB to include skin-associated bacteria as well as specific pathogens to represent both enteric and non-enteric pathogen risks. While FIB still have a role to indicate faecal contamination of greywater, it is important to also consider non-enteric pathogens (such as *S.* aureus and *P. aeruginosa*) and understand their fate and role in human health to ensure safe in-home greywater reuse. The research described in Chapters 3 and 4 should contribute to helping regulatory bodies better assess methods for testing treatment efficacy of greywater systems such as total staphylococci serving as an adequate endogenous performance surrogate to represent bacterial reductions (both enteric and non-enteric). It is recommended that further research should be performed to assess the efficacy of total staphylococci as an endogenous surrogate for multiple greywater sources and treatment methods compared to key pathogens of interest. In addition, it is recommended to attempt to better understand the fate of opportunistic and saprozoic bacteria throughout several greywater reuse scenarios by determining the effects of various disinfection methods on many of the aforementioned bacteria genera (*Staphylococcus*, *Corynebacterium*, *Propionibacterium*, and *Pseudomonas* spp.).

Chapter 4 showed that a PCP can significantly affect the performance of UV irradiation. Further research is needed to better understand both the range of PCP concentrations in greywater, as well as their effect on different disinfection methods. Our research indicated that at least partial removal of PCPs is necessary prior to UV irradiation for satisfactory disinfection. Further research should be carried out on the potential effects of a range of other PCPs on the efficacy of UV irradiation and various other disinfection methods; this knowledge will help to better understand the treatment needs for direct greywater reuse.

Chapter 5 showed that potentially pathogenic *S. aureus* could become enriched in a re-circulating greywater system using UV irradiation. It is possible this scenario may not persist in a real greywater reuse scenario, however further research is needed to determine the full implications of the results described in Chapter 5. These results may indicate the necessity for multiple disinfection steps to avoid enrichment of potentially pathogenic bacteria (such as *S. aureus* and

the saprozoic *P. aeruginosa*) within the greywater reuse cycle. To better understand the possibility of pathogenic bacteria becoming enriched in a greywater reuse system, it is recommended that the effects of cyclic disinfection (by UV and otherwise) on a range of bacteria (including other *Staphylococcus* spp., and saprozoic pathogens such as *P. aeruginosa*) be examined.

The bacterial composition of greywater has been shown to significantly differ between different sources; each greywater source and post-treated intended use poses unique challenges that must be assessed on an individual level. Hence, meaning *in situ* validation testing is necessary, for which total staphylococci may prove to be a most useful endogenous surrogate. Total staphylococci may be an adequate performance surrogate for bacterial, protozoan, and some virus reduction; however, it may not be adequate for all organisms. Although total staphylococci is consistently present in greywater at high concentrations (Benami *et al.* 2016, Zimmerman *et al.* 2014), this alone does not mean it would be an adequate measure for pathogen reduction for all microorganisms found in greywater, such as viruses, helminths, and fungal spores, given the range in UV resistance. In order to estimate relative log reduction targets between total staphylococci, viruses, fungal spores, and helminths, future studies need to explore the microbial composition of greywater in greater detail by quantifying more than just FIB.

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APPENDIX A: MICROBIAL CONCENTRATIONS IN GREYWATER

Table A.1: Bacteria of interest in greywater

Citation	(Benami *et al.* 2016)	(Zimmerman *et al.* 2014)	(Maimon *et al.* 2014)	(Siegrist 1977)	(Gilboa and Friedler 2007)	(Burrows *et al.* 1991)	(Casanova *et al.* 2001)	(Kim *et al.* 2009)
Reported Units	MPN/CFU	Log10 copies	CFU	CFU	CFU	CFU	CFU	CFU
GW Source	SH, WB	L	GW	WM	BT, SH, WB	SH	SH, WB, WM (No children)	GW
E. coli	1.9[b], (0.8[c] – 3.6[d])	1.0[a]	5.1[a] ± (5.5)[f], 4.3[b]	-	-	-	-	3.6[a]
***Enterococcus* spp.**	-	2.0[a]	-	-	-	-	-	-
Total *Bacteroides*	-	3.3[a]	-	-	-	-	-	-
Human-specific *Bacteroides*	-	2.7[a]	-	-	-	-	-	-
Total Coliforms	-	-	-	2.3[a], (1.8 – 2.8)[g]	-	>2	7.9[a] (5.8[c] – 8.3[d])	3.1[a]
Faecal Coliforms	-	-	-	2.0[a], (1.6 – 2.6)[g]	4.6[a] ± (5.0)[f]	-	5.8a (3.5c – 6.9d)	
***Staphylococcus* spp.**	2.7[b], (2.1[c] – 3.6[d])	6.5[a]	-	-	-	-	-	-
S. aureus	-	1.7[a]	3.7[a] ± (3.0)[f], <0[b]	ND[e]	4.0[a] ± (4.4)[f]	7.0[a] – 7.7[a]	ND[e]	3.3[a]
Corynebacterium	-	5.7[a]	-	-	-	-	-	-
Propionibacterium	-	5.4[da]	-	-	-	-	-	-
***Pseudomonas* spp.**	-	4.3[a]	-	-	-	ND[e]	-	-
P. aeruginosa	2.9[b], (2.0[c] – 4.5[d])	-	2.9[a] ± (3.2)[f], <0[b]	ND[e]	3.5[a] ± (3.5)[f]	-	5.0[a] ± (2.4[c] – 5.2[d])	-
HmtDNA	-	2.8[a]	-	-	-	-	-	-

Note: Not all organisms reported in the above studies are reported in this table
[a] Average, [b] Median, [c] Minimum, [d] Maximum, [e] Non-detect, [f] standard deviation, [g] 95% confidence interval
The examined studies sourced greywater from bathtubs (BT), mixed bathroom (BR), dishwasher (DW), mixed greywater (GW), mixed kitchen (K), kitchen sink (KS), laundry (L), laundry troughs (LT), personal washing (PW), showers (SH), washbasins (WB), and clothes washing machines (WM).

Table A.2: FIB in mixed source greywater

Mixed Greywater (or unreported source)						
Citation	GW Source	Reported Units	**Microbiological constituents**			
			Total coliforms	Faecal coliforms	*E. coli*	Enterococci
(Brandes 1978)	BT, K	N/A	8.1[a]	7.1[a]	-	
	GW	N/A	7.4[a], (4.8[c] – 8.1[d])	6.1[a], (3.7[c] – 7.3[d])	-	-
(Casanova *et al.* 2001)	SH, WB, WM (No children)	CFU	7.9[a] (5.8[c] – 8.3[d])	5.8[a] (3.5[c] – 6.9[d])	-	-
	SH, WB, WM (Children)	CFU	8.3[a]	7.0[a]	-	-
(Dallas *et al.* 2004)	GW	CFU	-	8.2[a] ± (8.7)[f]	-	-
(Friedler 2004)	BT, DW, KS, WB, WM	CFU	-	6.4[a]	-	-
(Friedler *et al.* 2005)	DW, KS, WM	CFU	-	5.7[a] ± (5.8)[f]	-	-
(Gerba *et al.* 1995)	BT, KS, SH, WB, WM	CFU	7.7[a]	6.8[a]	-	-
(Gross *et al.* 2005)	GW	CFU	-	6[a] ± (5)[f]	-	-
(Gross *et al.* 2008)	BT, K, L	CFU	-	3[a] – 6[a]	-	-
(Halalsheh *et al.* 2008)	GW (no KS)	MPN	7.5[a]	6.3[a]	5.8[a]	-
	KS	MPN	7[a]	5.5[a]	5.3[a]	-
(Jamrah *et al.* 2006)	L, SH, WB	MPN	2.6[a]	2.2[a]	-	-
(Jefferson *et al.* 2004)	BT, SH, WB	CFU	3.9[a] ± (4.0)[f]	-	3.3[a] ± (3.8)[f]	-
(Kim *et al.* 2009)	GW	CFU	3.1[a]	-	3.6[a]	-
(Maimon *et al.* 2014)	GW (w/ K)	CFU	-	-	5.2[a] ± (5.5)[f], 4.7[b]	-
	GW (no K	CFU	-	-	3.4[a] ± (3.8)[f], 2.4[b]	-
	GW	CFU	-	-	5.1[a] ± (5.5)[f], 4.3[b]	-
(Mandal *et al.* 2011)	BT, L, WB	CFU	4.6[a]	4.5[a]	4.6[a]	-
(Nolde 1999)	BT, SH, WM (w/ diapers)	MPN	4[c] – 6[d]	4[c] – 6[d]	-	-
(Ottoson and Stenström 2003)	GW	N/A	8.1[a] ± (0.78)[f], (5.5[c] – 8.7[d])	-	6.0[a] ± (0.60)[f], (4.3[c] – 6.8[d])	4.4[a] ± (0.48)[f], (3.0[c] – 5.1[d])
(Paris and Schlapp 2010)	K, L, SH	N/A	7.7[a]	5.8[a]	-	-
(Paulo *et al.* 2009)	BR, K, L	MPN	8.7[a] ± (8.8)[f]	-	6.7[a] ± (5.7)[f]	
(Rose *et al.* 1991)	GW (No children)	CFU	1.6[a]	1.6[a]	-	-
	GW (Children)	CFU	5.5[a]	3.2[a]	-	-
(Surendran and Wheatley 1998)	DW, L	CFU	6.7[a]	2.7[a]	-	-

Note: Not all organisms reported in the above studies are reported in this table
[a] Average, [b] Median, [c] Minimum, [d] Maximum, [e] Non-detect, [f] standard deviation, [g] 95% confidence interval
The examined studies sourced greywater from bathtubs (BT), mixed bathroom (BR), dishwasher (DW), mixed greywater (GW), mixed kitchen (K), kitchen sink (KS), laundry (L), laundry troughs (LT), personal washing (PW), showers (SH), washbasins (WB), and clothes washing machines (WM).

Table A.3: FIB in bathroom greywater

Bathroom Greywater						
Citation	GW Source	Reported Units	Microbiological constituents Total coliforms	Faecal coliforms	*E. coli*	Enterococci
(Beck *et al.* 2013)	BT, SH, WB	CFU	2.1[c] – 3.7[d]	-	-	1.8[c] – 3.0[d]
(Benami *et al.* 2016)	PW	MPN	-	-	1.9[b], (0.8[c] – 3.6[d])	-
(Birks and Hills 2007)	BT, SH, WB	CFU	7.3[a] ± 8.0	-	5.6[a] ± 6.4	3.4[a] ± 3.7
(Burrows *et al.* 1991)	SH	CFU	>2[c]	-	-	-
(Chaillou *et al.* 2010)	BT, SH	CFU	-	-	5.7[a] (4.4 – 6.3)[c]	5.5[a] (1.4 – 6.2)[c]
(Christova-Boal *et al.* 1996)	BT, SH	MPN	2.7[c] – 7.4[d]	2.2[c] – 3.5[d]	-	-
(Friedler 2004)	SH	CFU	-	6.6[a] ± (6.9)[f]	-	-
	WB	CFU	-	3.5[a] ± (3.9)[f]	-	-
	BT	CFU	-	6.6[a] ± (6.7)[f]	-	-
(Gilboa and Friedler 2007)	BT, SH, WB	CFU	-	4.6[a] ± (5.0)[f]	-	-
(Godfrey *et al.* 2010)	BT	CFU	4.2[c] – 5.4[d]	-	-	-
(Gual *et al.* 2008)	SH, WB	N/A	-	6[a]	-	-
(Halalsheh *et al.* 2008)	BT, WB	MPN	6.7[a]	4.7[a]	4.5[a]	-
(Jamrah *et al.* 2006)	SH	MPN	2.5[a]	1.8[a]	-	-
	WB	MPN	2.7[a]	2.4[a]	-	-
(Jefferson *et al.* 2004)	BT (Person 1)	CFU	3.8[a] ± (4.0)[f]	-	1.9[a] ± (2.1)[f]	-
	SH (Person 2)	CFU	4.2[a] ± (4.0)[f]	-	2.8[a] ± (3.2)[f]	-
	BT (Person 2)	CFU	4.4[a] ± (4.3)[f]	-	4.3[a] ± (4.3)[f]	-
	WB	CFU	4.0[a] ± (4.0)[f]	-	1[a] ± (3.9)[f]	-
	SH (Person 1)	CFU	3.8[a] ± (4.0)[f]	-	3.2[a] ± (3.7)[f]	-
(Katukiza *et al.* 2014)	BR	CFU	8[a] ± 7.8	-	6.4[a] ± 6.5	-
(Nolde 1999)	SH	MPN	1[c] – 3[d]	-1[c] – 1[d]	-	-
	BT, SH	MPN	2[c] – 3[d]	-1[c] – 1[d]	-	-
(O'Toole *et al.* 2012)	BR	MPN	-	-	3.2[a] ± (3.6)[f], 2.1[b], (ND[c, e] – 4.3[d])	-
(Pidou *et al.* 2008)	BT, SH, WB	MPN	4.8[a]	-	3.8[a]	3.4[a]
(Rose *et al.* 1991)	SH	CFU	5[a]	3.8[a]	-	-
(Siegrist 1977)	BT	CFU	3.3[a], (2.9 – 3.7)[g]	3.1[a], (2.7 – 3.5)[g]	-	-
(Siegrist *et al.* 1976)	BT, SH	CFU	2.0[a] ± (0.2)[f] (1.8[c] – 3.9[d])	2.3[a] ± (0.3)[f], (0[c] – 3.4[d])	-	-
(Surendran and Wheatley 1998)	PW	CFU	6.6[a]	2.4[a]	-	-
	BT, SH	CFU	6.8[a]	2.8[a]	-	-
	WB	CFU	4.7[a]	1.5[a]	-	-

Note: Not all organisms reported in the above studies are reported in this table
[a] Average, [b] Median, [c] Minimum, [d] Maximum, [e] Non-detect, [f] standard deviation, [g] 95% confidence interval
The examined studies sourced greywater from bathtubs (BT), mixed bathroom (BR), dishwasher (DW), mixed greywater (GW), mixed kitchen (K), kitchen sink (KS), laundry (L), laundry troughs (LT), personal washing (PW), showers (SH), washbasins (WB), and clothes washing machines (WM).

Table A.4: FIB in laundry greywater

Laundry Greywater						
Citation	GW Source	Reported Units	**Microbiological constituents** Total coliforms	Faecal coliforms	*E. coli*	Enterococci
(Christova-Boal *et al.* 1996)	LT, WM	MPN	3.4[c] – 5.5[d]	2.0[c] – 3.0[d]	-	-
(Friedler 2004)	WM	CFU	-	6.6	-	-
(Gross *et al.* 2008)	L	CFU	-	6[a], 4[b]	-	-
(Jamrah *et al.* 2006)	L	MPN	2.5[a]	1.1[a]	-	-
(Katukiza *et al.* 2014)	L	CFU	7.9[a] ± (7.8)[f]	-	6.2[a] ± (6.2)[f]	-
	WM (Wash)	MPN	-	-	5.0[a] ± (6.0)[f], 0.3[b], (ND[c, e] – 6.9[d])	-
	WM (Rinse)	MPN	-	-	3.5[a] ± (4.4)[f], 0[b], (ND[c, e] – 5.3[d])	-
(Rose *et al.* 1991)	L (Wash)	CFU	2.3[a]	2.1[a]	-	-
	L (Rinse)	CFU	1.7[a]	1.4[a]	-	-
(Siegrist *et al.* 1976)	WM	CFU	2.3[a], (1.8 – 2.8)[g]	2.0[a], (1.6 – 2.6)[g]	-	-
	L (Wash)	CFU	4.3[a] ± (0.4)[f], (1.9[c] – 5.9[d])	3.1[a] ± (0.3)[f], (1.0[c] – 4.2[d])	-	-
	L (Rinse)	CFU	3.7[a] ± (0.2)[f], (2.3[c] – 5.2[d])	2.5[a] ± (0.2)[f], (1.5[c] – 3.9[d])	-	-
(Surendran and Wheatley 1998)	WM	CFU	5.8[a]	2.9[a]	-	-
(Zimmerman *et al.* 2014)	L	Log_{10} copies.10 0 mL^{-1}	-	-	1.0[a]	2.0[a]

Note: Not all organisms reported in the above studies are reported in this table
[a] Average, [b] Median, [c] Minimum, [d] Maximum, [e] Non-detect, [f] standard deviation, [g] 95% confidence interval
The examined studies sourced greywater from bathtubs (BT), mixed bathroom (BR), dishwasher (DW), mixed greywater (GW), mixed kitchen (K), kitchen sink (KS), laundry (L), laundry troughs (LT), personal washing (PW), showers (SH), washbasins (WB), and clothes washing machines (WM).

Table A.5: FIB in kitchen greywater

Kitchen Greywater						
Citation	GW Source	Reported Units	Microbiological constituent			
			Total coliforms	Faecal coliforms	E. coli	Enterococci
(Brandes 1978)	K	N/A	7.9[a]	6.0[a]	-	-
(Friedler 2004)	KS	CFU	-	6.1[a] ± (6.4)[f]	-	-
	DW	CFU	-	4.8[a]	-	-
(Halalsheh *et al.* 2008)	KS	MPN	7.3[a]	6[a]	5.7[a]	-
(Katukiza *et al.* 2014)	K	CFU	7.8[a] ± (6.9)	-	ND[e]	-

Note: Not all organisms reported in the above studies are reported in this table
[a] Average, [b] Median, [c] Minimum, [d] Maximum, [e] Non-detect, [f] standard deviation, [g] 95% confidence interval
The examined studies sourced greywater from bathtubs (BT), mixed bathroom (BR), dishwasher (DW), mixed greywater (GW), mixed kitchen (K), kitchen sink (KS), laundry (L), laundry troughs (LT), personal washing (PW), showers (SH), washbasins (WB), and clothes washing machines (WM).

APPENDIX B: ADDITIONAL FIGURES AND TABLES

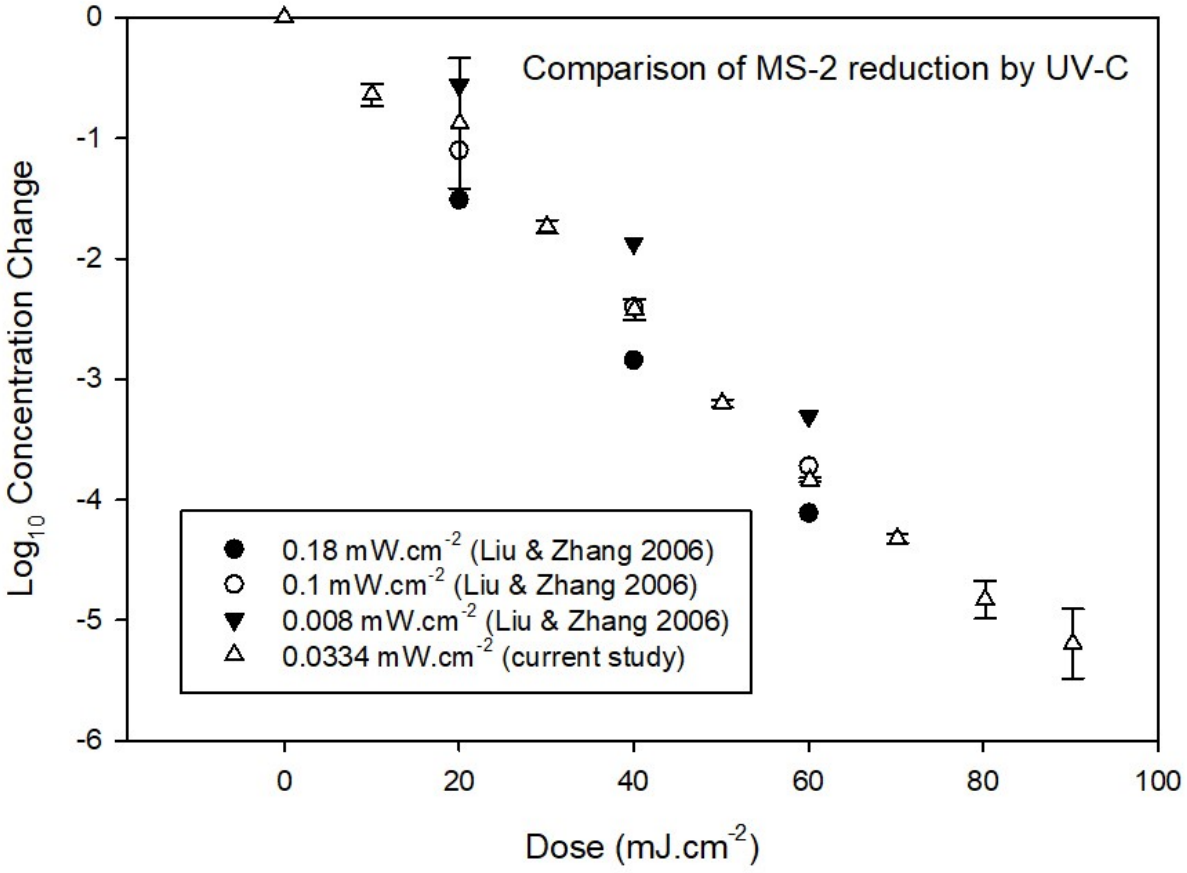

Figure B.1: MS2 Literature Comparison (Liu and Zhang 2006)

Table B.1: Raw hand-rinse water reduction

Sample	Dose (mJ.cm^{-2})	T-TSA Log CFU.100 mL^{-1}	SD	TS-MSA Log CFU.100mL^{-1}	SD	A_{256}
	0	6.36	5.12	6.35	5.38	
HW-1	220	3.77	3.06	3.74	3.13	0.360
	0	6.56	5.56	6.52	5.41	
HW-2	220	4.78	3.40	4.65	3.51	0.273
	0	5.68	4.70	5.39	5.14	
HW-3	220	3.48	2.69	3.02	2.51	0.164
	0	6.21	5.29	5.96	5.06	
HW-4	220	4.10	3.06	3.69	3.07	0.166
	0	6.26	5.26	6.01	5.05	
HW-5	220	4.19	3.06	3.98	3.19	0.249
Average	-	6.33	6.06	6.28	6.08	0.242

Table B.2: *Staphylococcus* spp. isolate data

***Staphylococcus* Isolate**	**Dose ($mJ.cm^{-2}$)**					
	7		**9**		**11**	
	LR	**SD**	**LR**	**SD**	**LR**	**SD**
S. aureus (ATCC 25923)	-		-	-	4.920819	0
S. aureus (i)	> 3.87	-	> 5.17	-	> 5.19	-
S. lentus (unknown)	-	-	-	-	5.05	0
S. pasteuri (iii)	-	-	-	-	5.817345	-
S. pasteuri (iv)	> 4.44	-	> 5.20	-	> 5.47	-
S. pasteuri (v)	-	-	-	-	5.07	0.06
S. capitis (ii)	-	-	-	-	> 5.70	-
S. capitis (iii)	4.34	0.27	> 6.30	-	> 6.30	-
S. haemolyticus (iv)	2.24	0.15	3.18	0.09	3.44	0.08
S. haemolyticus (v)	-	-	-	-	4.39	0.14
S. epidermidis (ii)	> 5.25	-	> 5.55	-	> 5.68	-
S. epidermidis (iiia)	-	-	-	-	> 6.09	-
S. epidermidis (iiib)	-	-	-	-	> 5.56	-
S. warneri (i)	3.30	0.16	> 4.81	-	> 5.71	-
S. warneri (ii)	-	-	-	-	> 5.55	-
S. *hominis* spp. *hominis* (v)	0.69	0.11	1.01	0.07	1.60	0.10

*Blue denotes insufficient plate count
*LR=Log reduction
Note: i-v denotes participant