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The Influence of Recycled Water on Microbial Source Tracking Techniques that Aim to Identify Untreated Sewage in Surface Waters

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by

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DEDICATION

This dissertation is dedicated to my family, including my loving father who has always pushed me to achieve every goal I set and showed me the value of hard work. To my grandmother, who has been the foundation of this family and has always cheered for my success. To tía Lulu, for being a role model by obtaining a PhD while raising my two amazing cousins. To tía Marisa, who I know is watching over me and always sending her love. To my wife Rosemery, for always being supportive of my goals and remaining a beacon of light in my life.

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ABSTRACT

Fecal pollution in recreational waters can introduce pathogens that cause increased human health risk, where exposure can occur during recreational activities such as swimming, diving, surfing, and fishing. Furthermore, human fecal pollution (i.e., sewage) can introduce nutrients and other harmful chemicals that may negatively affect the environment and aquatic species. Generally, sewage is considered the highest risk to human health compared to animal fecal pollution because it typically contains a high diversity of pathogens (including human specific viruses), antibiotic resistant bacteria and genes associated with antibiotic resistance. However, pathogens are difficult to detect due to their low concentrations in the environment, and there are too many to test every possible target. The use of surrogates that indicate the presence of pathogens is a standard approach that is utilized in recreational water quality studies. The most common surrogates are fecal indicator bacteria (FIB, e.g. enterococci and *Escherichia coli*) and their concentrations in water are used to estimate human health risk from contact with surface waters. One drawback to using FIB is that they do not provide information on the source of fecal contamination since humans and multiple animals can contribute to their concentration in surface waters.

Microbial source tracking (MST) DNA markers, which are frequently measured by quantitative PCR (qPCR) can alleviate limitations that arise from FIB methods. HF183, a DNA marker associated with sewage, can be measured in recreational waters to distinguish human sewage from other sources of fecal pollution by targeting a host-specific gene (i.e., HF183) in bacteria

strongly associated with sewage. However, recreational water quality studies that rely on qPCR alone lack the ability to distinguish viable intact cells from dead cells and extracellular DNA in surface waters. This limitation can lead to an overestimation of human health risk in recreational water quality studies that rely on analysis of DNA. This issue is particularly acute in cases when treated, disinfected sewage (e.g., recycled water), which is known to contain microbial DNA, is directly discharged into surface waters and could be incorrectly identified as untreated sewage contamination.

This dissertation aims to address the following knowledge gaps, i) the effectiveness of different levels of wastewater treatment on the reduction of DNA in recycled water, ii) the decay rate of DNA from recycled water vs. untreated sewage in recreational waters, and the usefulness of culturable EcH8 as a viable marker of sewage pollution, and iii) the extent to which recreational water quality methods approved by regulatory agencies such as the U.S. Environmental Protection Agency capture extracellular DNA. In Chapter Two, we examined the effect of recycled water discharge on DNA marker levels in a Florida stream and tested the persistence of sewage-associated markers (i.e., HF183, H8, and CPQ_056) from wastewater treatment facilities that have two different levels of treatment. Recycled water from an advanced wastewater treatment (AWT) facility was discharged into a Florida stream and increased concentrations of the sewage-associated HF183 marker 1000-fold. Persistence of sewage-associated microorganisms was compared by qPCR in untreated sewage and recycled water from conventional wastewater treatment (CWT) and AWT facilities in Tampa and St. Petersburg, Florida. Multivariate analysis found that the persistence of sewage-associated DNA markers (HF183 and crAssphage CPQ_056) were significantly greater following CWT compared to AWT. Differential decay of DNA markers was found in recycled water samples where bacterial

markers HF183 and EC23S857 were significantly correlated with each other but were not correlated to the viral marker CPQ_056. We tested to see if culturable EcH8 can be used to distinguish untreated sewage from recycled water and examined the proportion of total *E. coli* that carry the H8 gene. The proportion of total *E. coli* that carried the sewage associated H8 gene (culturable EcH8) in untreated sewage ranged from 8 – 18%, while culturable *E. coli* were below the limit of detection (< 1 CFU/L) in all recycled water samples. Therefore, culturable EcH8 has potential to confirm the presence of untreated sewage in surface waters that also contain DNA from recycled water.

In Chapter Three, the persistence of sewage-associated DNA markers (HF183 and CPQ_056) in outdoor freshwater mesocosms that were spiked with recycled water or untreated sewage and sampled over a five-day period were compared by qPCR. The persistence of culturable EcH8 was also measured to assess how it compared to sewage-associated DNA markers and to determine if it would be a useful target for detecting sewage over time. Experiments were conducted on three separate trials in a shaded environment to simulate a Florida stream. On day 5, median $\log_{10}$ reduction of sewage-associated DNA markers in the recycled water treatment were 0.68 (HF183) and 0.44 (CPQ_056), and were 2.83 (HF183), and 1.0 (CPQ_056) in the sewage treatment. The persistence of DNA markers assessed by multivariate analysis was significantly greater in the recycled water treatment compared to the untreated sewage treatment. The relationship between light intensity and decay rate of microbial variables was significant. In the sewage treatment, culturable EcH8 was detected in 40 to 60% of samples after five days across the three trials but was undetectable in recycled water. These results demonstrate the environmental persistence of DNA from recycled water and support the usefulness of culturable

EcH8 for detecting untreated sewage in recreational waters that are also impacted by recycled water and other disinfected discharges of wastewater.

The objective for Chapter Four was to use standard recreational water quality methods to determine the proportion of DNA from intact cells and extracellular DNA (exDNA) that can be captured on filters and subsequently detected by qPCR, while exploring the usefulness of DNase I to eliminate extracellular DNA on membranes. Intact cells and extracellular DNA (obtained by boil lysis) from pure cultures of the FIB *E. coli* or *Enterococcus faecalis*, and exDNA from *Natronomonas pharaonis* (used as an exogenous control for river water and recycled water) were concentrated by membrane filtration. DNA was extracted from the filter and the proportion captured was measured with qPCR genetic markers EC23S857 (*E. coli*), Entero1A (*Ent. faecalis*), and NP*gyr*A (*N. pharaonis*). For intact cells, the proportion of gene copies captured on membranes ranged from 80 – 86% and were not significantly different among EC23S857 and Entero1A markers, DNase I treatment did not negatively affect gene copy estimates for intact cells. For solutions of exDNA, the mean percentage of EC23S857 gene copies captured on membranes was 1.4% and was significantly greater than Entero1A (0.5%). For river water and recycled water spiked with exDNA from *N. pharaonis*, the mean percentage of gene copies captured was 0.62% and 1.32%, respectively. DNase I treatment of membranes significantly reduced exDNA in all sample types by ~ 2 $\log_{10}$. These data demonstrate that a low percentage (< 2%) of exDNA in environmental water can be captured by standard recreational water quality methods; however, high concentrations of exDNA in water samples could complicate interpretations of data based on DNA measurements. DNase I treatment of membranes is a useful strategy to alleviate exDNA interference and could be used to improve estimates of human health risk in recreational waters.

This work will benefit human and ecosystem health by providing information and tools that could improve identification of untreated sewage pollution in recreational waters. Knowledge gained from this research can expand the recreational water quality field by highlighting important limitations to standard methods and help prevent overestimations of human health risk.

CHAPTER ONE: BACKGROUND AND REVIEW OF FECAL INDICATOR BACTERIA, MICROBIAL SOURCE TRACKING MARKERS, METHODS THAT ACCOUNT FOR EXTRACELLULAR DNA, AND WASTEWATER TREATMENT

Fecal Contamination and Microbial Source Tracking Techniques

Recreation in surface waters (e.g., oceans, lakes, and rivers) often includes activities such as swimming, kayaking, and fishing, while one 2012 estimate found that more than 140 million people recreate in water bodies in the U.S. (1). Fecal contamination, including sewage pollution, contributes nutrients to surface waters, which can cause eutrophication and harmful algal blooms (2). This type of anthropogenic pollution can also limit the beneficial uses of surface water. For example, recreation and products from aquaculture may be harmful to human health when high levels of fecal bacteria and pathogens are present (3). Furthermore, elevated levels of harmful microorganisms can lead to beach closures, which can be an economic burden for areas that rely on revenue from tourists (4). Gastrointestinal (GI) illness is a major health concern for recreational swimmers exposed to fecal pollution in contaminated surface waters (5-6). For example, a recent study estimated that roughly 90 million illnesses occur annually in the United States due to human exposure to fecal pollution in surface waters, with an economic burden of roughly $2.2 to $3.7 billion each year (7).

One hundred and forty waterborne disease outbreaks (defined as an epidemiological association between time/location of recreational water exposure and the event of two or more closely related illnesses) associated with exposure to recreational waters were reported to the Centers for

Disease Control and Prevention from 2000 - 2014 (8). Pathogens implicated in these outbreaks included protozoa (e.g., *Cryptosporidium* spp., *Giardia intestinalis*), bacteria (e.g., *Campylobacter jejuni*, *Escherichia coli* O157:H7, *Shigella sonnei*), and viruses (e.g., adenovirus and norovirus) (8-9). Recent outbreaks associated with recreational water exposure included *S. sonnei* (California), norovirus (Maine), and Shiga toxin-producing *E. coli* (Minnesota) which were reported between 2018 and 2019 (10). It is important to note that most waterborne diseases occur sporadically and many individuals do not seek medical care, therefore these diseases are underreported (8). These pathogens and other potentially harmful microorganisms, including antibiotic resistant bacteria, can be deposited into surface waters through feces from domestic (pets and livestock) and wildlife animals (e.g., deer and birds) and human sources such as leaks/overflows of untreated sewage, poorly functioning septic systems, and wastewater discharge (11-14). Microorganisms from multiple sources can also be introduced to surface waters through urban or agricultural stormwater runoff (15-16).

Monitoring every possible pathogen in surface waters is complex and is currently not feasible, due to their low concentrations and high diversity in the environment, and their specific growth requirements (17). Alternative methods have therefore been established as surrogates for pathogens in order to predict human health risk from exposure to pathogens (18-20). FIB are widely accepted surrogates for monitoring pathogens, since several epidemiological studies revealed a strong correlation between levels of FIB in surface waters and the frequency of GI illness cases in recreational swimmers (5-6, 21). The U.S. Environmental Protection Agency (EPA) published guidelines for monitoring fecal pollution by measuring culturable (viable) concentrations of FIB such as fecal coliforms (18), and more recently shifted to the use of *E. coli* (19) and enterococci (20) in recreational waters. These methods use membrane filtration to

concentrate bacteria and measure concentrations of viable FIB. The EPA has also established recreational water quality criteria for a qPCR method that measures DNA from *Enterococcus* spp. (Entero1A) in recreational surface waters (22).

Regulatory agencies with oversight ranging from local (e.g., Environmental Protection Commission of Hillsborough County) to international (e.g., World Health Organization) currently use FIB concentrations as a non-specific indicator of fecal pollution from all possible sources. However, many known limitations of FIB as predictors of human health risk exist, including their infrequent correlation with the presence of pathogens when waters are impacted by non-human sources of fecal pollution (23-26). FIB, including *E. coli* and enterococci can originate from the gastrointestinal tract of multiple hosts, e.g., humans, dogs, turtles, alligators, deer, birds, cattle, swine and poultry (Figure 1.1) (27-33). Different sources of fecal pollution have varying potential to contribute pathogens to recreational waters (34). Human fecal pollution (i.e., untreated sewage) is generally considered a high risk to human health because it typically contains a high diversity of pathogens, including human-specific viruses (35-37). Furthermore, a high diversity of genes associated with antibiotic resistance are known to be present in untreated sewage and may increase human health risk if acquired by pathogens (38).

Sources of fecal pollution can vary in health risk to humans; therefore, an accurate prediction of human health risk from exposure to fecal contaminated surface waters requires precise identification of sources and estimates of the extent of contamination from each source. One risk assessment study found that exposure to recreational waters contaminated with human or cattle feces is significantly more likely to cause gastrointestinal illness compared to waters polluted by other domestic or wild animal sources (39). Epidemiological studies have revealed that the ability to distinguish among sources of fecal pollution is imperative to improve predictions of

health risk in contaminated surface waters (40-43). Consequently, microbial source tracking (MST) techniques were developed to identify the most likely host species contributing to fecal pollution in impacted water bodies. Many current MST methods utilize quantitative polymerase chain reaction (qPCR) techniques to measure specific genes of host-associated bacteria or viruses that are shed in feces and are predominantly associated with one host species. *Bacteroides dorei*, which carries the HF183 genetic marker, is one example of a bacterium that is strongly associated with the human gastrointestinal tract and is ubiquitous in untreated sewage (44). A genetic marker such as HF183 can help estimate the extent of human fecal pollution and provide evidence to guide remediation efforts, as demonstrated in previous studies (45-46). MST methodology has provided the means to distinguish among sources of fecal pollution, and to provide evidence of major non-point sources to watershed managers (47).

MST methods, like FIB methods, typically employ membrane filtration to concentrate and cultivate bacteria from water samples. A recently developed assay that targets the sewage-associated crAssphage CPQ_056 genetic marker (48), offers a novel tool to further improve MST efforts by utilizing a viral surrogate that is at high concentrations in sewage to help discriminate sewage from non-human fecal sources. MST assays targeting various animal fecal sources have also been developed and are applied across the world to track fecal pollution from domestic/wildlife animals including birds (GFD) (49), cows (CowM2 and CowM3) (50), dogs (DG37) (51), horses (HoF597) (52), pigs (Pig2Bac) (53), poultry (LA35) (54), and others not included in this list. For example, one study in Florida provided evidence that high levels of fecal indicator bacteria were largely attributed to avian species in the watershed by measuring levels of HF183 and the avian-associated GFD marker (47). This result helped managers avoid costly remediation strategies such as total maximum daily load (TMDL) programs for this watershed.

Scientists are expanding their ability to effectively differentiate sources of fecal pollution with MST tools at their disposal; however, these methods have limitations as they typically rely entirely on the use of qPCR to detect DNA markers. An important limitation of qPCR methods is their inability to discriminate between viable and nonviable cells, since they simply detect nucleic acid (i.e., DNA and RNA). Treated wastewater contains extracellular nucleic acids from lysed microbes, which enters surface waters by pathways such as direct discharge or irrigation runoff (55-56). Studies found in the literature demonstrated that molecular signatures (e.g. MST markers and genes associated with pathogens or antibiotic resistance) are detected even in highly-treated recycled water (38, 57-61); therefore, qPCR methods alone cannot distinguish treated wastewater from untreated sewage as the source of fecal contamination in environmental waters. Thus, reliance on genetic markers that survive wastewater treatment and enter surface waters can misinform regulatory agencies and lead to failed remediation efforts.

Implications of Extracellular DNA for MST Studies and Possible Solutions

Sewage is typically disinfected with chlorine or ultraviolet (UV) light to inactivate any bacteria or viruses that survive the initial treatment stages before the final product (effluent) enters recycled water distribution systems or is discharged to environmental waters. A key difference between untreated sewage and recycled water is that FIB (e.g., *E. coli* and enterococci) are generally dead following disinfection. However, the persistence of DNA following wastewater treatment and disinfection, and the extent to which the DNA is captured by membrane filtration of surface waters remains a knowledge gap in the literature. Prevention and cessation of untreated sewage leaks and spills is a high priority for regulatory agencies, but human exposure to recycled water is not generally considered a health risk (62-65). Differentiating recycled water from untreated sewage is necessary since exposure to recycled water constitutes a much lower

health risk than exposure to untreated sewage, which contains more viable pathogens. Sewage has far different ramifications for management strategies while recycled water is currently not a concern.

Extracellular DNA is a target of interest for many studies, primarily in the field of ecology, where it is generally termed “environmental DNA” (66-69). Conversely, detection of DNA, which has no association with human health risk, can be a disadvantage while attempting to make an association to infectious pathogens as done with viable surrogates (e.g. FIB) in the recreational water quality field. Recreational water quality methods typically rely on filtration through membranes containing pores of a defined diameter (membrane filtration) to concentrate microorganisms from water. In theory, DNA should pass through these filters, but some fraction of extracellular DNA is captured on membranes (70). Retention of extracellular DNA (*C. parvum* 18S rRNA gene fragment) by membrane filtration was demonstrated in one study with qPCR, where membrane composition and water quality parameters (e.g., total suspended solids and pH) had the greatest influence on the recovery (maximum 18% of initial DNA) of extracellular DNA (70). Detection of extracellular DNA in environmental waters can be misleading and contribute to an overestimation of health risk due to the measurement of DNA from dead bacterial cells, e.g., such a scenario can occur with recycled water exposure. A deeper understanding of the extent to which extracellular DNA is captured by protocols used for recreational water quality assessment is crucial for interpretation of qPCR data derived from surface waters suspected of being contaminated with human and/or animal feces.

Culture-based methods, coupled with molecular analysis to detect bacteria associated with sewage, can improve efforts to distinguish inputs from sewage vs. disinfected wastewater effluent and recycled water in environmental waters. One such example is a real-time PCR

method based on genes in *E. coli* associated with human feces, which were identified by whole genome sequencing of *E. coli* isolates from a variety of fecal sources (71). Marker performance studies with culturable *E. coli* containing these genes were conducted in Japan (71), in Australia (72), and in the U.S. (73). The U.S. study found the H8 gene (sodium/hydrogen exchanger precursor) to have the highest sensitivity (percentage of sewage and human fecal samples positive for the targeted gene) and specificity (percentage of non-human animal fecal samples negative for the sewage-associated gene) among the four genes tested by qPCR for assessing human sewage in Florida surface waters (73). PCR methods used in these studies can be adopted to test viable *E. coli* cells for the sewage-associated H8 gene and distinguish untreated sewage from persistent DNA in the environment.

Several studies have explored other methods to distinguish qPCR signals from dead and viable cells, but each method has limitations. Ethidium monoazide (EMA) or propidium monoazide (PMA) treatment prior to qPCR has been proposed as a method of live/dead discrimination, as these photoreactive DNA-binding dyes preferentially bind to extracellular DNA and DNA in cells with compromised membranes, thereby interfering with the PCR reaction (74). However, some studies reported no significant differences in variables measured by qPCR with or without EMA/PMA treatment (75-76). The effectiveness of EMA treatment for live/dead discrimination varies between Gram-positive and Gram-negative bacteria (74). In some cases, EMA and PMA penetrate viable cell membranes, leading to an underestimation of live cells (77-79), while in two different studies PMA treatment failed to inhibit qPCR amplification from dead cells and led to an overestimation of viable bacteria (74, 80). Studies on EMA/PMA treatment are generally conducted on pelleted bacteria or include multiple washing steps for membranes, and therefore provide little insight on the efficacy of this approach when bacteria are concentrated on

membranes. Bonetta et al. (2017) demonstrated the use of PMA treatment on membrane filters with *Legionella pneumophila* cells and found that PMA efficiency was dependent on low concentrations of *L. pneumophila* in water, presenting a challenge for environmental applications (81). The persistent qPCR signal following EMA/PMA treatment in disinfected wastewater or recycled water in these studies, could be attributed to the persistence of intact cells that carry the qPCR target genes, or other method limitations described above. To our knowledge, few studies have utilized these methods to differentiate the qPCR signal from intact cells and extracellular DNA in recycled water or disinfected wastewater effluent (75, 82-83).

Other examples of methods proposed for viability discrimination include quantitative reverse transcriptase PCR (qRT-PCR), inversely-coupled immunomagnetic separation and adenosine triphosphate (Inv-IMS/ATP) quantification, and DNase I treatment prior to qPCR. Studies show that qRT-PCR methods, which are based on quantification of messenger RNA, are complex and have limitations in environmental matrices (84-86). Furthermore, mRNA expression varies among genes, while also shifting with phases of cell growth (84-86). On the other hand, Inv-IMS/ATP assays measure ATP production in specific microbes e.g., *Bacteroides thetaiotaomicron*; however, specificity of this method can decrease in water that is highly contaminated by feces of host species other than the target, while sensitivity can be negatively affected in samples with elevated turbidity (87). Although DNase I treatment prior to qPCR aims to eliminate extracellular DNA and DNA from dead cells in samples, the efficacy of this method is limited by environmental factors that can inhibit enzyme activity (88-89). Two other studies have demonstrated the ability to circumvent inhibitors in environmental samples by conducting DNase I treatment directly on membrane filters with concentrated bacteria and report high enzyme efficacy (90-91).

Wastewater Treatment and Recycled Water

The primary purpose of sewage treatment for over a century has been to mitigate pollution of water bodies with nutrients and pathogenic microorganisms (92). Municipal wastewater treatment facility (WWTF) processes in the U.S. currently focus on decreasing inorganic nitrogen, total phosphorus, organic carbon (measured by reduction of biological and chemical oxygen demand), and pathogens (measured by reduction of FIB) (93). The U.S., according to past estimates, produces roughly 32 billion gallons per day of municipal wastewater (56). Only 7-8 % of effluent is further treated to produce recycled water for urban, industrial, environmental, and agricultural purposes (56). However, treatment and disinfection methods for recycled water that reduce levels of microorganisms and their genes in the effluent can vary widely among WWTFs within the same geographic area, including disinfection methods (e.g., chlorination, ozonation, pasteurization, UV) which are prevalent in the U.S (56).

WWTFs are characterized by different levels of treatment and are typically classified as conventional or advanced. Conventional wastewater treatment (CWT) facilities in the U.S. always include a primary stage which relies on physical processes to settle solids, followed by a secondary biological treatment stage, then disinfected by UV or chlorine before discharge. Advanced wastewater treatment (AWT) processes are designed to further reduce concentrations of nutrients and microbial contaminants in secondary-treated sewage effluent (94). AWT can include a variety of processes that can decrease levels of pathogens (e.g., biological aerated filter, flocculation, membrane filtration, chemical oxidation, , membrane bioreactor, dual media), nutrients (e.g., adsorption, denitrification filters), or both pathogens and nutrients (e.g., five-stage Bardenpho processes) (94-97). Compared to CWT facilities, AWT can lower concentrations of pathogens (e.g., *E. coli*, enterovirus, *Cryptosporidium*, norovirus, *Giardia* and *Salmonella*) and

antibiotic resistant bacteria in disinfected effluents (97-101). However, recycled water can be produced by CWT and AWT facilities, thus different levels of treatment result in varying concentrations of microorganisms and their genes in the treated effluent (60, 102-103). The effect of CWT vs. AWT on the persistence of MST markers through wastewater treatment remains a knowledge gap and should be further explored to understand the fate of sewage-associated markers and its possible implications for recreational water quality studies.

Among the important challenges facing the recreational water quality and MST fields are: i) the extent to which recycled water distribution systems allow target genes associated with sewage, pathogens, and antibiotic resistant bacteria to enter the environment, and ii) limitations of qPCR techniques to distinguish DNA in recycled water from the untreated sewage qPCR signal in environmental waters. Recently, Florida was recognized as the national leader for recycled water usage and it is estimated that approximately 820 million gallons per day are being used for beneficial applications (104). In 2014, estimates showed that within the Southwest Florida Water Management District (Figure 1.2) approximately 151 million gallons of recycled water was used per day (105). The study reported that over 100,000 residential customers, 9,000 acres of predominately citrus crops, and about 200 golf courses relied on recycled water for irrigation (105). Average rainfall for counties in this district was approximately 40 - 60 inches per year (106), which means that surface waters are prone to impact from large amounts of stormwater runoff that could contribute recycled water to surface waters. On the other hand, environmental waters can receive millions of gallons of recycled water or treated effluent per day by direct discharge from WWTPs, which is more likely to interfere with estimates of human health risk than stormwater runoff (60).

Research Chapters: Dissertation Objectives

Chapter 2: Persistence of Sewage-Associated Genetic Markers in Advanced and Conventional Treated Recycled Water: Implications for Microbial Source Tracking in Surface Waters

Rationale: Sewage-associated DNA markers used for microbial source tracking (e.g. HF183 and CPQ_056) may also be present in recycled water if DNA persists through wastewater treatment. In this case, dead bacteria or free DNA from recycled water may provide a similar signal to viable culturable bacteria from sewage in surface waters, leading to inaccurate identification of the source of contamination and overestimation of health risk. One key difference found between sewage and reclaimed water is the presence of viable/culturable bacteria. Therefore, a feasible solution to the problem of discriminating sewage from recycled water could be to culture *E. coli* and test for the sewage-associated H8 marker. It is also important to understand how advanced vs. conventional treatment impacts the fate of MST markers in recycled water and its potential to interfere with MST efforts designed to identify untreated sewage in surface waters.

Methodology: Differences in MST marker gene concentrations were examined by qPCR and their persistence were compared by frequency of detection and $\log_{10}$ reduction (decay) in untreated sewage and recycled water from three AWT and three CWT facilities in Florida, U.S.A. Culturable *E. coli* were isolated from untreated sewage and recycled water from each facility to test for the H8 genetic marker.

Hypotheses: The hypotheses tested are: (i) recycled water contains levels of sewage-associated MST marker genes that could influence MST analysis of fecal sources in surface waters, (ii) persistence of sewage-associated MST genes in recycled water is reduced in AWT compared to CWT facilities and (iii) culturable *E. coli* containing the H8 gene are consistently present in untreated sewage and absent in recycled water.

Chapter 3: Differential Persistence of Microbial Source Tracking Genetic Markers in Recycled Water Compared to Untreated Sewage in a Freshwater Environment

Rationale: The decay rates of MST markers through wastewater treatment were previously established in Chapter 2. However, DNA in recycled water could be more susceptible to decay from abiotic/biotic factors in the environment compared to DNA in intact cells from sewage. Furthermore, little is known about the persistence of culturable *E. coli* with the H8 gene in surface waters, which impacts this method's ability to detect untreated sewage in surface waters over time. This study will provide data on the extent to which the qPCR signal in recycled water can complicate the interpretation of efforts to identify untreated sewage, and the usefulness of culturable *E. coli* with the H8 gene for identification of sewage in surface waters.

Methodology: Outdoor mesocosms composed of river water spiked with recycled water or untreated sewage were constructed to compare the decay of one bacterial and one viral DNA marker of sewage, as well as a general FIB (*E. coli*) DNA marker in a shaded outdoor environment. The presence of viable H8-positive *E. coli* (culturable EcH8) was also assessed over time in the mesocosms.

Hypotheses: Two major hypotheses were tested in this study: (i) DNA markers detected by qPCR will decay more rapidly in environmental water spiked with recycled water compared to the same DNA markers in samples spiked with sewage, and (ii) the persistence of culturable EcH8 in the water mesocosms will be comparable to that of the DNA markers.

Chapter 4: Does Extracellular DNA From Treated Wastewater Have Potential to Influence Microbial Analyses of Recreational Water Quality?

Rationale: The qPCR signal of fecal microorganisms observed in recycled water could be extracellular DNA captured on membrane filters using protocols for recreational water quality

methods. If extracellular DNA is a contributing factor, then methods (i.e., DNase I treatment) may be adopted to eliminate this qPCR signal and avoid false identification of untreated sewage in surface water samples.

Methodology: Capture of intact cells and extracellular DNA from pure cultures of *Escherichia coli* or *Enterococcus faecalis* were quantified by qPCR through membrane filtration and DNA extraction with DNA markers EC23S857 and Entero1A. Extracellular DNA from *Natronomonas pharaonis* (extremely haloalkaliphilic archaeon), which is not found in river water, was spiked into recycled water and river water to test how much extracellular DNA was captured by quantifying the DNA marker NP*gyr*A. DNase I treatment was conducted on membranes to assess the possibility of elimination of the qPCR signal derived from extracellular DNA.

Hypotheses: This study included two hypotheses: (i) a fraction of extracellular DNA can be captured and quantified by standard recreational water quality techniques, and (ii) DNase I treatment eliminates the persistent qPCR signal from free DNA in recycled water or river water.

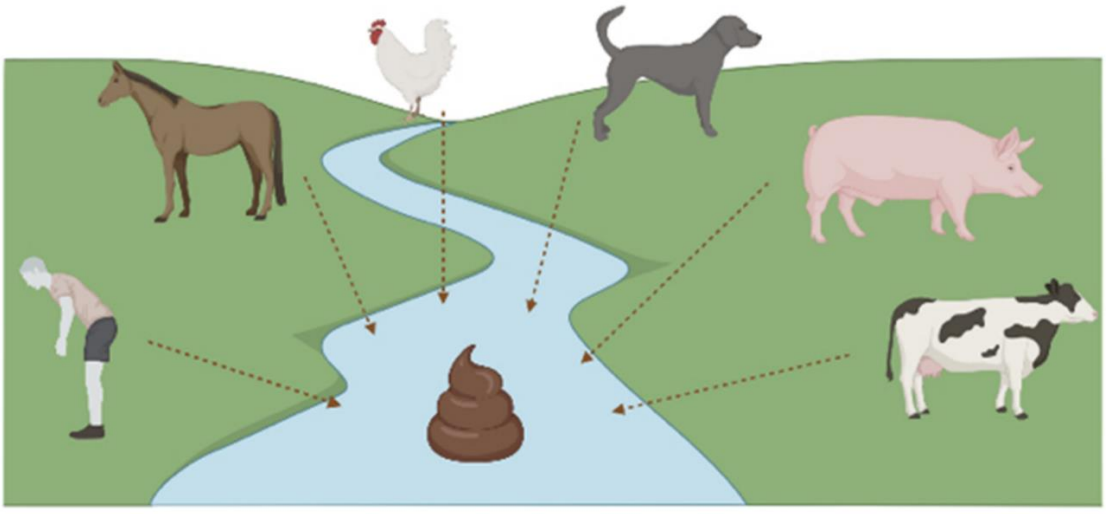

Figure 1.1. Humans and animals can contribute FIB to surface waters making it difficult to identify the source of contamination (image created with BioRender.com).

Figure 1.2. Map of Southwest Florida Water Management District (107).

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CHAPTER TWO: PERSISTENCE OF SEWAGE-ASSOCIATED GENETIC MARKERS IN ADVANCED AND CONVENTIONAL TREATED RECYCLED WATER: IMPLICATIONS FOR MICROBIAL SOURCE TRACKING IN SURFACE WATERS

[This chapter has been previously published, see Appendix A.]

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Abstract

Sewage contamination of environmental waters is increasingly assessed by measuring DNA from sewage-associated microorganisms in microbial source tracking (MST) approaches. However, DNA can persist through wastewater treatment and reach surface waters when treated sewage/recycled water is discharged, which may falsely indicate pollution from untreated sewage. Recycled water discharged from an advanced wastewater treatment (AWT) facility into a Florida stream elevated the sewage-associated HF183 marker 1000-fold, with a minimal increase in cultured *Escherichia coli*. Persistence of sewage-associated microorganisms was compared by qPCR in untreated sewage and recycled water from conventional wastewater treatment (CWT) and AWT facilities. *E. coli* (EC23S857) and sewage-associated markers HF183, H8, and viral crAssphage CPQ_056 were always detected in untreated sewage (6.5 – 8.7 $\log_{10}$ GC/100 mL). Multivariate analysis found significantly greater reduction of microbial variables via AWT vs CWT. Bacterial markers decayed ~4-5 $\log_{10}$ through CWT, but CPQ_056 was ~100-fold more persistent. In AWT facilities, $\log_{10}$ reduction of all variables was ~5. In recycled water, bacterial marker concentrations were significantly correlated ($p \leq 0.0136$; tau $\geq$ 0.44); however, CPQ_056 was not correlated with any marker, suggesting varying drivers of decay. Concentrations of cultured *E. coli* carrying the H8 marker (EcH8) in untreated sewage were 5.24-6.02 $\log_{10}$ CFU/100 mL, while no *E. coli* was isolated from recycled water. HF183 and culturable EcH8 were also correlated in contaminated surface waters (odds ratio $\beta_1 = 1.701$). Culturable EcH8 has strong potential to differentiate positive MST marker signals arising from treated (e.g., recycled water) from untreated sewage discharged into environmental waters.

Importance

Genes in sewage-associated microorganisms are widely accepted indicators of sewage pollution in environmental waters. However, DNA can persist through wastewater treatment, and reach surface waters when recycled water is discharged, potentially causing indications of untreated sewage pollution. Previous studies have found that bacterial and viral sewage-associated genes persist through wastewater treatment; however, these studies did not compare different facilities or identify a solution to distinguish untreated sewage from recycled water. In this study, we demonstrate the persistence of bacterial markers and the greater persistence of a viral marker (CPQ_056 of crAssphage) through advanced and conventional wastewater treatment scenarios. We aimed to provide a tool to confirm the presence of untreated sewage contamination in surface waters with recycled water inputs. This work showed that the level of wastewater treatment affects the removal of microbial genetic markers, particularly viruses, and expands our ability to identify treated and untreated sewage in surface waters.

Introduction

Untreated sewage pollution can introduce waterborne pathogens, which are a major health concern for recreational swimmers, into surface waters (1-3). Discrimination of fecal sources has become a priority since human fecal pollution (i.e., untreated sewage) is considered a higher risk to human health compared to other fecal sources due to the presence of a high diversity of pathogens, including human specific viruses (4, 5). If the source of pollution is not identified, remediation efforts and attempts to mitigate further pollution will likely fail. To combat the deficiencies of conventional fecal indicator bacteria such as *E. coli*, which cannot discriminate among fecal sources and provide insufficient information about human health risk, microbial source tracking (MST) methods based on host-associated genetic markers have been developed

(6). For example, the bacterial HF183 MST marker is strongly associated with the human gastrointestinal tract and is ubiquitous in untreated sewage in the United States (7). Measuring microbial variables such as HF183 by qPCR can also help estimate human health risk by measuring the extent of human fecal/sewage pollution and distinguish contamination from human feces and untreated sewage from domestic or wildlife animal sources (7). One limitation with this approach is that qPCR methods lack the ability to discriminate between viable and nonviable cells, as well as free DNA, since they simply detect nucleic acid.

Differentiating recycled water from untreated sewage in surface waters is necessary for accurate risk assessment since epidemiological studies have shown a lower health risk from exposure to recycled water (8-12), while exposure to untreated sewage is a definite health risk for recreational water users (13, 14). The risk associated with exposure to recycled water by direct potable reuse is highly dependent on the treatment process utilized and the density of pathogens present in sewage prior to treatment (15). Approximately 12 – 15 log reduction of viruses is recommended for safe direct potable reuse of treated wastewater (i.e., recycled water), however, there is a need for more accurate methods that measure viral infectivity for risk assessments (16). In recreational waters, the reduction of viruses in recycled water required for safe conditions may be lower (16); however, the required level of treatment is unclear. Despite this knowledge gap, it remains imperative that we can distinguish untreated sewage from recycled water in the environment to prioritize sources of fecal microorganisms in environmental waters and better inform future epidemiology studies and risk assessments.

Recycled water containing quantifiable levels of sewage-associated MST markers may interfere with MST efforts in environmental waters focused on the identification of only untreated sewage. In addition to discharge into surface waters, recycled water can be redirected and used

for irrigation, groundwater recharge, or other applications (17). Recycled water applications with strong potential to impact surface water, such as lawn irrigation, are prevalent in many states in the U.S., including Florida. An estimated minimum of 900 million gallons per day of recycled water is utilized by Florida alone in various land applications such as edible crops (6,000 acres), 500 golf courses, 1,000 schools, and 500,000 residences (18). Recycled water that enters surface waters can contribute DNA from compromised or dead cells to the environment (19, 20). Previous studies have found that two sewage-associated viral genetic markers (crAssphage and pepper mild mottle virus), the bacterial marker HF183, and antibiotic resistance genes persist through production of recycled water (19, 21-23).

Recycled water can be produced in advanced or conventional wastewater treatment facilities (WWTFs), which vary in the level of treatment. Conventional wastewater treatment (CWT) facilities in the U.S. typically employ primary (physical) and secondary (biological) treatment, followed by disinfection. Advanced wastewater treatment (AWT) can include multiple approaches to reduce levels of nutrients and microbial contaminants in secondary-treated sewage effluent, e.g., adsorption, dual media filters, denitrification filters, membrane filtration, membrane bioreactor, flocculation, biological aerated filter, chemical oxidation, and Bardenpho processes (24, 25). Persistence through wastewater treatment is generally reported by measuring the frequency of detection or decay (e.g., $\log_{10}$ reduction) of microbial analytes. Previous studies demonstrated that AWT can further reduce concentrations of pathogens (e.g., *Cryptosporidium*, *E. coli*, enterovirus, *Giardia*, norovirus and *Salmonella*) and antibiotic resistant bacteria (24, 26-29) compared to CWT. However, the literature lacks evidence for the relative efficacy of removal of sewage-associated genetic markers in AWT versus CWT facilities. A deeper understanding of these differences is necessary to guide MST efforts and to inform decisions on

the treatment process used to produce the recycled water that ultimately enters environmental surface waters.

Improving methods to quantify infectious pathogens in surface waters is an ongoing effort that is crucial to accurate health risk assessments, which frequently rely on measuring surrogates by qPCR. No widely-accepted method to eliminate DNA, and thus the qPCR signal, from dead cells in treated wastewater has been established. Some studies have utilized techniques (e.g., propidium monoazide treatment, PMA) that attempt to prevent amplification of DNA from non-viable cells in qPCR tests (30, 31); however, constituents such as total suspended solids can interfere with light activation of the PMA dye, and a major knowledge gap remains on the dye's ability to penetrate cell membranes of viable but non-culturable cells (32). Even if these techniques could fully attenuate PCR amplification in viable but non-culturable cells, they lack the ability to eliminate a qPCR signal originating from dead cells or free-DNA (extracellular) (33), further confounding the live/dead interpretation. A method that combines cultivation and genetic techniques to quantify sewage-associated bacterial genetic markers may provide a solution for the discrimination of untreated sewage from recycled water in treated waste flows and environmental surface waters.

Viable *E. coli* is consistently present in untreated sewage and is generally not culturable in recycled water. Four gene fragments in *E. coli* strains associated with human feces were previously identified by whole genome sequencing (34). A performance study of culturable *E. coli* containing these genes was conducted in the U.S. and found that the H8 gene, a sodium/hydrogen exchanger precursor, had the highest sensitivity (percentage of target, i.e., sewage and human fecal samples, positive for the targeted gene) and specificity [percentage of non-target (fecal samples from animals other than human) samples negative for sewage-

associated gene] among the four genes tested for tracking untreated sewage pollution in sub-tropical surface waters (35). These methods may have utility to identify viable *E. coli* cells originating from untreated sewage without confounding target DNA from extracellular and dead cells in recycled water by including an enrichment step to test *E. coli* isolates for the H8 marker.

We tested three major hypotheses in this study: the first two aimed at exploring the potential for recycled water to produce positive indications of untreated sewage pollution in environmental waters tested by qPCR methods, and the third tested an alternative method based on culture of *E. coli* followed by probe-based real-time PCR to detect the H8 gene (culturable EcH8). The hypotheses addressed are: (i) recycled water contains levels of sewage-associated MST marker genes that could influence MST analysis of fecal sources in surface waters, (ii) persistence of sewage-associated MST genes in recycled water are reduced in AWT compared to CWT facilities and (iii) culturable *E. coli* containing the H8 gene is consistently present in untreated sewage and absent in recycled water. To test these hypotheses, we examined differences in MST marker gene concentrations and compared their persistence by frequency of detection and $\log_{10}$ reduction in untreated sewage and recycled water from three AWT and three CWT facilities in Florida, U.S.

Methods

Sites and Sampling

Three types of experiments were carried out in this study. We examined (i) the effect of recycled water discharge on levels of HF183 and culturable *E. coli*, (ii) persistence of MST markers through AWT and CWT, and (iii) conducted a surface water survey to assess the relationship between HF183 and culturable EcH8. In experiment (i), the effect of the input of recycled water discharged from an AWT facility on culturable *E. coli* and HF183 levels in Turkey Creek, a first-

order Florida stream, was evaluated during a discharge event. Samples were collected seven days before, during, and 23 h after a scheduled recycled water discharge from three sites: the discharge point (latitude: 27.955679, longitude: -82.20926), from which water flows along a canal into the creek, a site 1.21 km downstream which was impacted by the discharged effluent, and a site 0.24 km upstream of the confluence and is not affected by the discharge (Figure B.1). Water samples were collected in sterilized, 1 L polypropylene bottles, transported on ice, and processed within two hours.

In experiment (ii), untreated sewage and recycled water were collected between March and September 2021 from three AWT (facility D - F) and CWT (facility A - C) facilities in Tampa and St. Petersburg, Florida (Table 2.1). Each WWTF location was sampled three times, yielding 18 untreated sewage samples and 18 recycled water (treated) samples. Untreated sewage (500 mL) and recycled water (2 L) samples were collected in sterile polypropylene containers and transported at 4 °C on wet ice to the laboratory. Samples were processed within 6 hours for analysis of culturable *E. coli* and DNA extraction.

In experiment (iii), surface water samples from eight water bodies in St. Petersburg, Florida were collected monthly for two years. These sites are classified as impaired waterbodies due to the consistent exceedance of recreational water quality criteria for fecal indicator bacteria levels, which may have been influenced by untreated sewage inputs. The area was also serviced by irrigation lines delivering recycled water. Samples (500 mL) were stored up to 2 hours in sterile 500 mL Nalgene containers on wet ice and processed by membrane filtration for cultivation of *E. coli* and environmental DNA extraction.

E. coli Culture

Surface water samples were concentrated in duplicate using three volumes (0.1 mL, 1 mL, and 10 mL) onto mixed cellulose ester filters (47 mm diameter 0.45 µm pore size; Fisherbrand) by membrane filtration. *E. coli* was cultured from the samples and enumerated on mTEC utilizing USEPA Method 1603 (36). In addition, a phosphate buffered saline (PBS, pH 7.0) blank was filtered and plated on mTEC to check for contamination. Prior to each sampling event, mTEC agar was tested with a positive control (*E. coli*, ATCC 11775™) and a negative control (*Enterococcus faecalis*, ATCC 19433™). *E. coli* concentrations were reported in CFU/100 mL. The limit of detection for culturable *E. coli* in surface waters was 1×10^{1} CFU/100 mL.

Bacteria from untreated sewage and recycled water were concentrated by membrane filtration as described above. Untreated sewage samples were diluted 10^{-3} -fold, and 1 mL was filtered (equivalent to 0.001 mL of untreated sewage). One liter of each recycled water sample was concentrated by filtration. *E. coli* was cultured and enumerated as described above. The limit of detection for culturable *E. coli* in this study was 1.0×10^{5} CFU/100 mL for untreated sewage, and 1×10^{-1} CFU/100 mL for recycled water.

DNA Extraction for Microbial Analysis

For all surface water samples, 500 mL of each water sample was filtered through a Fisherbrand 47 mm mixed cellulose ester membrane with 0.45 µm pores. Membrane filters were aseptically folded and placed in Qiagen PowerBead Tubes and stored at -80 ºC (< 1 month). Untreated sewage samples (10 mL) were mixed with 990 mL of phosphate buffered saline (PBS; pH 7.4) in a sterile beaker for 1 min with a magnetic stirrer then concentrated by membrane filtration as described above. Recycled water was directly filtered to concentrate 1 L by membrane filtration as described above. DNA extractions were performed on all water samples using the Qiagen

Dneasy PowerWater™ Kit using manufacturer's instructions. QPCR for MST markers was performed as described in the section below. One hundred µL of purified DNA was eluted for all samples in this study.

QPCR Analysis of Microbial Variables

QPCR was conducted to quantify sewage-associated *Bacteroides* HF183 following USEPA method 1696 (7) in surface water and WWTF samples, a general *E. coli* target EC23S857 (37), sewage-associated H8 (35), and crAssphage CPQ_056 (38) was also tested on DNA extracted from AWT and CWT facilities. QPCR amplification was conducted in 25 µL reactions in triplicate using 12.5 µL TaqMan Environmental Master Mix 2.0 (Applied Biosystems) and 5 µL of template DNA per reaction in a Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories; California, US). All assays included 40 thermal cycles, and primer and probe concentrations/sequences and qPCR assay conditions are reported in supplementary material (Table B.1). Standard curves were constructed from synthetic gene fragments (gBlocks™, Integrated DNA Technologies) (Table B.2) containing the target sequences and reference DNA material (inhibition amplification control, HF183 only) was included for each sample according to guidelines in USEPA method 1696 (7). All standard curves ranged from 10^7 to 5 gene copies per reaction. Performance metrics included efficiencies between 90 % and 110 %, and R^2 values ranging from 0.979 for EC23S857 to 0.998 for HF183. In untreated sewage samples, the limit of detection was 500 GC/100 mL and the limit of quantification was 1,000 GC/100 mL for each qPCR assay. For recycled water samples, the limit of detection was 10 GC/100 mL and the limit of quantification was 20 GC/100 mL for each qPCR assay. Inhibition of qPCR amplification was not detected in any of the samples tested in this study (data

not shown). Negative controls for each instrument run included 4 extraction blanks and 4 non-template controls which were all negative for each qPCR target in this study.

Molecular Analysis of Culturable EcH8

Isolated colonies with characteristic *E. coli* morphology were individually picked with a sterile toothpick from mTEC agar plates for culturable EcH8 analysis. Each colony was suspended in 50 µL of reagent grade water, and boiled for 10 min at 100 °C in a thermal cycler as described in a previous study (35). PCR amplification of an *E. coli* specific β-glucuronidase *uid*A gene was conducted to confirm colonies as *E. coli* (39). Confirmed colonies (30 per sample) extracted by boiling lysis were also individually tested for presence of the H8 gene by PCR. PCR amplification was conducted in 25 µL reactions that included 12.5 µL TaqMan Environmental Master Mix 2.0 (Applied Biosystems) and 5 µL of template DNA per reaction in a Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories; California, US). Primer and probe concentrations/sequences and PCR assay conditions are reported in supplementary material (Table B.1). A genomic positive control for *uid*A and H8, ATCC 13706™ was included in each run for comparison. An H8 negative control (ATCC 11775™) was also included in each run.

Data Analyses

R version 4.1.3 (40) was used for statistical analyses in this study which included descriptive statistics, hypothesis testing, and correlation analyses. A significance threshold of $\alpha < 0.05$ was set for all statistical tests. LOQ was defined as the lowest standard concentration that consistently amplified in all three replicates, while LOD was the lowest standard concentration in which at least 2 out of 3 replicates consistently amplified. Data that were below the LOQ and the LOD were considered censored and values were calculated for statistical analyses (41). In this study,

left-censored data was only in recycled water samples and censored observations were only below the LOD, this data was expressed as a range from 0 to LOD – 1. To account for censored observations in microbial data u-scores were calculated based on v 1.2 of u-score script written by D. Helsel (available in PracticalStats.com) (41). U-scores were utilized to compute statistical analyses that tested for relationships between microbial variables and differences by treatment type. All data were $\log_{10}$-transformed prior to statistical analysis.

Descriptive statistics (median and standard deviation) were calculated by treatment type (AWT or CWT) and sample type (untreated sewage or recycled water) for culturable *E. coli* and qPCR (EC23S857, HF183, H8, CPQ_056) data through the R package stats (version 4.1.3). The nonparametric Kruskal-Wallis one-way analysis of variance by ranks test was executed for univariate analysis of significant differences in microbial variables in untreated sewage and recycled water from AWT compared to CWT facilities. Differences among microbial variables were determined by Kruskal-Wallis rank test followed by pairwise comparisons using the Dunn's test for multiple comparisons in untreated sewage and recycled water data through the R package rstatix (version 0.7.2), with the Benjamini & Hochberg method used for p-value correction. Left-censored microbial data for recycled water samples included qPCR-derived measurements (n=18) of HF183 (22%), H8 (33%), and CPQ_056 (22%). Robust regression on order statistics was implemented via the R package NADA (version 1.6-1.1) to compute medians and interquartile ranges for left-censored data (41, 42). The frequency of detection was determined for each microbial variable and compared across treatment type (AWT and CWT) in untreated sewage or recycled water data sets with a Fisher exact test (43) and the R package rstatix (version 0.7.2), with the Benjamini & Hochberg method used for p-value correction. Proportions of culturable *E. coli* with the H8 gene (culturable EcH8) were compared across wastewater

treatment facilities, significant differences were determined though the Fisher exact test (43). Relationships between microbial variables were analyzed with Kendall's rank correlation tau, where the coefficient (tau) can range from -1.0 to 1.0. A value of -1.0 designates a perfect negative correlation between two variables; a value of 1.0 indicates a perfect positive correlation. A value of 0 demonstrates that no linear relationship exists between two variables.

(i) Effects of advanced vs conventional treatment on concentrations of microbial variables

A two-way permutational multivariate analysis of variance (PERMANOVA) was executed with the vegan R package (44), to determine if there was a significant effect of treatment type (AWT or CWT) on all concentrations of fecal (EC23S857) and sewage indicators measured (HF183, H8, and CPQ_056) in recycled water. The homogeneity of multivariate dispersion assumption was met prior to PERMANOVA among groups (treatment type). Linear discriminant analysis (LDA) was performed to visualize variability in measurements among AWT and CWT facilities, LDA was accomplished by constructing an ordination plot through a canonical analysis of principal coordinates via the Biodiversity R package (45).

(ii) Analysis of the relationship between HF183 and culturable EcH8 in surface waters

HF183 concentrations were $\log_{10}$ transformed and compared to the frequency of culturable EcH8 detection by binary logistic regression. Detection of culturable EcH8 was defined as 1.0 if there was amplification of DNA from at least 1 isolate out of the 30 tested for a given sample, or 0.0 was assigned if 0 out of 30 isolates amplified for culturable EcH8. Binary logistic regression was carried out using GraphPad Prism version 10.0.2. to determine log-likelihood, odds ratio, and the confidence interval around the odds ratio.

Data availability

Data are located in the University of South Florida (USF) Digital Commons (https://digitalcommons.usf.edu) at DOI: 10.17632/5dh58k5x2f.1.

Results

Discharge Study: Impact of Recycled Water on HF183 Concentrations in a Florida Stream

A Florida stream (Figure B.1) that receives one to six million gallons of recycled water during sporadic discharge events was sampled to measure HF183 concentrations prior to discharge, during a known discharge event, and after discharge (Table 2.2). The stream on average is approximately 5.15 meters wide and 0.4 meters deep with an average flow of 0.26 meters/sec. HF183 was detected at the discharge outfall site at low concentrations (1.3 $\log_{10}$ GC/100 mL) prior to the discharge and was not detected at the upstream site nor the downstream site (Table 2.2). During discharge, HF183 concentrations increased by approximately 3 orders of magnitude in the discharge outfall (4.15 $\log_{10}$ GC/100mL) and at the downstream site (3.58 $\log_{10}$ GC/100mL), while HF183 was not detected in the site upstream of the outfall (Table 2.2). After discharge, HF183 was only detected at the downstream site and was about 2 orders of magnitude lower than what was recorded during the recycled water discharge event (1.63 $\log_{10}$ GC/100mL) (Table 2.2). During effluent discharge, concentrations of culturable *E. coli* at the discharge site were reduced to < 1 CFU/100 mL and were about 3.31 $\log_{10}$ CFU/100 mL at the downstream site, but the concentrations at both sites returned to their previous concentration the next day (Table 2.2).

Association of Facility Type (AWT and CWT) with Microbial Variables in Untreated Sewage and Recycled Water

We compared microbial variables by qPCR in untreated sewage and recycled water from AWT vs CWT facilities. For untreated sewage, the concentration and frequency of detection of microbial variables was measured. Concentrations of crAssphage (CPQ_056) and the general *E. coli* marker gene EC23S857 were significantly greater in untreated sewage from AWT compared to CWT facilities, while HF183 and H8 concentrations were not significantly different (Figure 2.1, P-values in Table B.3). Multivariate analysis of all microbial variables showed that concentrations were significantly greater in untreated sewage collected from AWT facilities compared to CWT facilities (Figure B.2). All microbial variables were quantifiable in each untreated sewage sample from AWT and CWT facilities.

In recycled water samples, we compared concentrations of qPCR marker genes and their persistence (frequency of detection or $\log_{10}$ reduction) between AWT and CWT facilities. Concentrations of crAssphage CPQ_056 and the H8 marker (2.12 and 0.99 $\log_{10}$ GC/100 mL, respectively) were significantly lower in recycled water produced by AWT facilities compared to CWT facilities (5.67 and 1.52 $\log_{10}$ GC/100 mL, respectively), while no significant difference was observed for EC23S857 and HF183 (Figure 2.2, P-values in Table 2.3). Canonical analysis of principal coordinates demonstrated a clear separation of microbial variables in recycled water produced in AWT compared to CWT facilities (Figure 2.3). CPQ_056 followed by the H8 marker were the variables that best explained differences in concentrations of microbial variables in recycled water produced by AWT and CWT facilities (Figure 2.3). HF183 and H8 markers were significantly less frequently detected in recycled water from AWT facilities (44 % and 33 %, respectively) compared to CWT facilities (100 %) (Tables 2.3 and 2.4), while crAssphage

CPQ_056 showed a similar trend but was not significant (p = 0.0824; Table 2.3). Multivariate analysis of all microbial variables showed that concentrations were significantly greater in recycled water from CWT facilities compared to AWT facilities (Table 2.3).

The persistence of each microbial variable was further explored by comparing decay rates (log_{10} reduction) from untreated sewage to recycled water in AWT compared to CWT facilities, as this metric accounts for initial concentration in influent as well as final concentration in recycled water. Univariate analysis showed that decay rates of all microbial variables were somewhat greater in AWT facilities compared to conventional treatment (Figure 2.4), although only CPQ_056 experienced significantly greater log_{10} reduction of 5.50 in AWT facilities compared to 1.82 in CWT facilities (Figure 2.4, Table 2.3). For all microbial variables, the multivariate analysis showed a significantly lower persistence (frequency of detection and log_{10} reduction) in AWT compared to CWT facilities (Table 2.3). All log_{10} reductions values measured in this study are available in supplemental material (Table B.4).

Differences Among Microbial Variables: Pooled Data from AWT and CWT Facilities

We pooled data from all facilities to focus on differences among concentrations of microbial variables irrespective of treatment strategies. In untreated sewage, all microbial variables measured by qPCR were detected and quantifiable in each untreated sewage sample tested during this study. *E. coli* EC23S857 marker concentrations (8.14 log_{10} GC/100 mL) were the highest followed by HF183 (7.76 log_{10} GC/100 mL), crAssphage CPQ_056 (7.63 log_{10} GC/100 mL), and H8 marker (6.95 log_{10} GC/100 mL) (Figure 2.1). EC23S857 marker concentrations were significantly greater than all other variables except HF183 (P-values in Table B.5), although the comparison was on the verge of significance (P = 0.0512). The concentrations of all other microbial variables were significantly greater than the H8 marker (Table B.5). HF183 and

crAssphage CPQ_056 concentrations were not significantly different in pooled untreated sewage data (Table B.5).

In recycled water, we examined which markers were most dominant and prevalent. We pooled data of microbial variables measured by qPCR and compared differences among microbial variables using three metrics: concentration, frequency of detection, and $\log_{10}$ reduction. The median concentration of CPQ_056 (4.71 $\log_{10}$ GC/100 mL) was ranked the highest followed by EC23S857 (2.42 $\log_{10}$ GC/100 mL), HF183 (2.23 $\log_{10}$ GC/100 mL), and H8 marker (1.26 $\log_{10}$ GC/100 mL). CPQ_056 concentrations were significantly greater than that of H8 marker but were not significantly different from HF183 or EC23S857 (Table B.5). No other comparisons of median values in recycled water were significant (Table B.5). Comparison of frequency of detection (Table 2.4) found that EC23S857 (100 %) was significantly greater than HF183 (72 %) and the H8 marker (67 %) in pooled recycled water data (Table B.5). CPQ_056 frequency of detection (78 %, Table 2.4) was not significantly different from that of any of the bacterial variables (EC23S857, HF183, and H8) (Table B.5). Median $\log_{10}$ reduction values (Table B.4) (~2.8 - 5.76 $\log_{10}$) were not significantly different among qPCR marker genes when all recycled water data were pooled (Table B.5).

We also examined relationships among microbial variables measured by qPCR in untreated sewage or recycled water to compare differences in their removal in pooled datasets from AWT and CWT facilities. Significant relationships among microbial variables were found in untreated sewage data, i.e., concentrations of the H8 marker were positively correlated with EC23S857, HF183, and crAssphage CPQ_056 while EC23S857 levels positively correlated with CPQ_056 concentrations (Figure B.3, P-values in Table B.6). HF183 concentrations were not correlated with levels of EC23S857 or CPQ_056 (Table B.6). In recycled water, significant positive

correlations were found among concentrations of all bacterial variables (HF183, H8 marker and EC23S857) (Figure B.4, Table B.6). No relationship was found between CPQ_056 concentrations and any bacterial variables (EC23S857, HF183, and H8 marker) in all recycled water data (Figure B.4, Table B.6).

Culturable E. coli and Culturable EcH8 in Untreated Sewage and Recycled Water

Concentrations of culturable *E. coli* and the proportion carrying the H8 gene were compared across AWT and CWT facilities in untreated sewage and recycled water samples. Culturable *E. coli* concentrations in untreated sewage were not significantly different among all WWTFs, ranging from 6.46 to 6.67 $\log_{10}$ CFU/100 mL (Table 2.5, Table B.3). Culturable EcH8 was detected in all untreated sewage samples, and estimated concentrations of culturable EcH8 obtained by multiplying total *E. coli* concentration by the percentage of colonies positive for H8 ranged from 5.24 to 6.02 $\log_{10}$ CFU/100 mL. Culturable EcH8 in untreated sewage comprised ~ 14% of *E. coli* colonies tested over the duration of the study. The frequency of culturable EcH8 in the culturable *E. coli* population ranged from 8 to 18% at the various WWTFs and was not significantly different in untreated sewage from AWT compared to CWT facilities (Table 2.5, Table B.3). No *E. coli* were detected in recycled water samples (< 0.1 CFU/100 mL, n=18) over the duration of the study, therefore, no culturable *E. coli* could be tested for the H8 gene in recycled water.

HF183 and Culturable EcH8 Relationship in Surface Water Survey

Surface water samples from the St. Petersburg area characterized by chronically elevated fecal indicator bacteria levels contained HF183 levels ranging from below LOD to 4.31 $\log_{10}$ GC/100 mL. Culturable EcH8 was detected in 16.5% of 103 surface water samples (Table B.7). HF183 was detected in 82.5% of these samples and in 94.1% of the 17 samples that were positive for

culturable EcH8 (Table B.7). HF183 concentration and culturable EcH8 detection were positively and significantly correlated by logistic regression ($P = 0.0354$) (Figure 2.5). The odds ratio of the logistic regression was $\beta_1 = 1.701$ with a 95% confidence interval of 1.068 to 2.921.

Discussion

Recycled water production for urban, industrial, environmental, and agricultural applications in the U.S. is estimated to rise from 4.8 to 6.6 billion gallons per day by 2027 according to a Bluefield research survey (46). Recycled water delivered to surface waters can be misidentified as untreated sewage due to the persistence of sewage-associated markers such as HF183 and crAssphage (CPQ_056) through wastewater treatment (22, 47, 48). The persistence of other sewage-associated MST markers through wastewater treatment and in environmental waters is less understood, and a comparative study of MST marker persistence in AWT and CWT facilities has, to our knowledge, not previously been described. We have noted the potential for the influence of recycled water on the occurrence of MST markers associated with untreated sewage at many sites in Florida and were able to directly demonstrate it in a discharge event from an AWT facility described in this study. This work has advanced the MST field by showing that persistence of sewage-associated markers was dependent on the treatment level and was shown to be significantly lower in AWT vs. CWT recycled water by multivariate analysis. It also provides novel data that demonstrates the effect of recycled water discharge from an AWT facility on MST markers in surface water, and the use of culturable EcH8 in viable *E. coli* to discriminate between untreated sewage and recycled water.

Persistence and Levels of Sewage-Associated MST Marker Genes in AWT and CWT Facilities

Multiple environmental and experimental design factors influence the observed variability of microorganisms and their genes in treated wastewater effluent (reviewed in 49). Variable

persistence of microbes and their DNA can be influenced by factors such as treatment strategy utilized (50), air temperature, and elevation (51). Within a facility, the time of day the sample is collected (52), flow rate (53), and seasonal effects (54) can all influence microbial concentrations. Post- secondary treatment stages in AWT facilities sampled here include anoxic basins and oxidation ditches, dual media deep-bed denitrification filters, activated sludge treatment in an anoxic/aerobic configuration, and a five-stage Bardenpho activated sludge process, each of which could feasibly contribute to reduction of DNA in recycled water. All facilities in this study utilized chlorination for disinfection except AWT facility D, which only used ultraviolet light for disinfection. AWT facility D performed poorly in terms of DNA reduction and was also the only AWT facility with recycled water that contained detectable and quantifiable levels of all qPCR markers for each sample tested in this study. High concentrations of HF183 and the H8 marker in recycled water produced by AWT facility D led to non-significant differences in $\log_{10}$ reduction of MST markers between AWT and CWT facilities. In general, differences among WWTFs complicate generalizations about treatment efficacy, and the issue is exacerbated in AWT facilities, where many treatment options exist (e.g., Table 2.1). Studies of microbial persistence through wastewater treatment should therefore include salient details about treatment processes to maximize the usefulness of the data.

Multivariate and univariate analysis of microbial variables in AWT and CWT facilities showed lower concentrations and greater persistence of most microbial variables measured by qPCR in AWT facilities. Univariate analysis of individual variables revealed significantly lower levels and greater decay of crAssphage CPQ_056 in AWT compared to CWT facilities by all metrics; in fact, CPQ_056 $\log_{10}$ reduction in CWT recycled water was only ~2 $\log_{10,}$ compared to ~ 5.5 $\log_{10}$ in AWT recycled water. To the best of our knowledge, only one other study has reported

qPCR measurements of a sewage-associated indicator in recycled water produced by AWT and CWT facilities. Morrison et al. (55) found that the frequency of detection of CPQ_056 was 43% in recycled water produced in a AWT facility compared to 76% from a CWT facility in Arizona (U.S.). These findings agree with the data from this study; however, any broad generalizations must await further studies.

Several studies report MST marker persistence in WWTFs, measuring DNA targets such as HF183 and crAssphage (e.g., CPQ_056) however, they did not compare data from AWT compared to CWT facilities (22, 56, 57). Several studies conducted in the U.S. measured HF183 in disinfected effluent, finding less effective reduction compared to our study (58, 59). Two AWT facilities sampled in the U.S. produced recycled water containing HF183 in 100% of samples, and $\log_{10}$ reduction values ranged from 2.0 - 4.2 (58), whereas HF183 in recycled water from AWT facilities in our study was detected in only 44% of samples, with $\log_{10}$ reduction values from 4.1 – 6.9. Removal of HF183 in recycled water from a CWT facility was two orders of magnitude lower than CWT facilities tested in our study (~3 compared to ~ 5 $\log_{10}$ reduction (59). This study (59) used only 100 mL sample sizes and acidified the sample prior to filtration, which may have lowered recovery of the bacterial HF183 gene target. Another study conducted in North-East England detected HF183 and HumM2 (sewage-associated genetic marker) in 100% of disinfected effluent samples and median $\log_{10}$ reductions ranged from 1.3 to 1.4 for the 15 WWTFs tested, however, this study included 12 small treatment facilities and did not provide AWT or CWT classification (54). These few papers form the probing edge of our knowledge about persistence of sewage-associated markers in AWT and CWT facilities. What is clear is that bacterial and viral MST marker DNA persists through AWT and CWT facilities in the U.S. and

in other countries, which could confound the identification of untreated sewage in surface waters when recycled water is present.

Relative Persistence of Sewage-Associated MST Markers Through Wastewater Treatment

Very few comparative studies of the persistence of bacterial and viral MST marker DNA in recycled water have been performed. We found that crAssphage CPQ_056 was markedly more persistent than other microbial variables measured in this study, particularly in CWT facilities, where we observed ~2 $\log_{10}$ reduction, in contrast to ~5 $\log_{10}$ reduction of the bacterial variables measured by qPCR. Furthermore, no relationship between CPQ_056 concentrations and those of any bacterial variable was observed in recycled water, however, bacterial variables were all correlated, suggesting different drivers of decay between viral and bacterial targets. A study conducted at a CWT facility (Indiana, USA) found similar persistence of CPQ_056 (2.88 $\log_{10}$ reduction) compared to HF183 (3.33 $\log_{10}$ reduction) in disinfected effluent, in contrast to our study; however, only one facility was sampled (59). Other sewage indicators such as human adenovirus (HAdV) and human polyomaviruses (HPyVs) were significantly more persistent than HF183 and CPQ_056 in a U.S. study, with mean $\log_{10}$ reductions of 3.33 (HF183), 2.88 (CPQ_056), 2.24 (HAdV) and 1.51 (HPyVs) in disinfected effluent (59). These few studies demonstrate that persistence of sewage indicators varies in different wastewater treatment facilities and that the ability to distinguish between treated and untreated sewage in surface water applications could represent a major advance.

Differential persistence of sewage-associated microbes through wastewater treatment was noted in this study. Variability in persistence of bacteria can be attributed in part to cellular physiology e.g., tolerance to low nutrients and temperature (60). However, DNA from sewage-associated viruses tends to display greater persistence than bacterial DNA through wastewater treatment

(22, 57), which can be explained in part by size; viruses are smaller than bacteria and are not consistently removed in settling tanks (reviewed in 61). Furthermore, viruses are generally not as susceptible as bacteria to chemical disinfectants (62, 63). One challenge in utilizing viral markers (e.g., HAdV and HPyVs) to estimate persistence of viruses is that viruses are generally several orders of magnitude lower than concentrations of HF183 in untreated sewage and may not be detectable in diluted effluents (64). CrAssphage DNA, on the other hand, is present at higher concentrations than other viral marker genes in untreated sewage and was more persistent through wastewater treatment than any bacterial variable in this study, which further supports its use as an indicator for viral persistence in WWTFs. Understanding the differential persistence of MST marker genes through wastewater treatment and their concentration in disinfected effluent or recycled water will further support the selection of optimal markers for different research and regulatory applications.

H8 in Culturable E coli: Confirmation of Untreated Sewage Contamination when Recycled Water is a Confounding Factor

This study has shown that testing DNA extracted from surface waters for H8 via qPCR is fraught with the same issues as other qPCR assays, i.e., DNA persists through wastewater treatment and presents the same limitations as other methods such as HF183 and crAssphage. The H8 sequence has also been reported in *Klebsiella* and *Yersinia* strains that are not associated with the human gastrointestinal tract, (34, 65), hence, optimal strategies should involve cultivation and isolation of *E. coli* prior to H8 testing. Accurate identification of untreated sewage in surface waters that are also impacted by recycled water can be supported by a confirmatory step to avoid positive indications of untreated sewage and overestimation of health risk that can arise from sole reliance on nucleic acid-based methods. Utilizing a method that relies on detection of viable

bacteria that are also associated with human feces and untreated sewage is one possible path. Because culturable *E. coli* are at such low levels in recycled water that they are rarely detected (this study; 20, 66), amplification of the sewage-associated H8 gene in viable *E. coli* (culturable EcH8) can be applied to circumvent the limitations inherent in qPCR-based testing for untreated sewage when treated wastewater is present. The surface water survey in this study found a significant positive relationship between culturable EcH8 detection and HF183 concentration in water bodies impacted by untreated sewage and recycled water around Tampa Bay, Florida.

Another advantageous characteristic of the culturable EcH8 assay is the widespread use of *E. coli* as a fecal indicator for contamination of water and food (35, 67). Culturable *E. coli* is a commonly-used regulatory tool for assessment of recreational water quality (36) and many other aspects of sanitation, including wastewater treatment, therefore many facilities in the U.S. and other countries have the capacity to quantify *E. coli* by culture methods. The culturable EcH8 method in viable *E. coli* can also be utilized to identify the presence of human fecal contamination in other applications such as household or food industry studies that test surfaces and food to estimate human health risks.

The proportion (8 – 18% in this study) of culturable *E. coli* with H8 in untreated sewage varied across WWTFs, suggesting the need to test untreated sewage at facilities near a site of interest prior to embarking upon a study. Specificity of culturable EcH8 for human/sewage sources in previous studies ranged from 92 – 99% in Australia (65), Japan (34), Thailand (68), and the U.S. (35). Culturable EcH8 was detected in all untreated sewage samples in this study and in a previous U.S. study (35). Lower sensitivity was reported when reference samples included individual fecal samples, i.e. in Australia at 45% (65), Japan at 30% (34), and Thailand at 36% (68), suggesting that the culturable EcH8 genetic marker is not shed in all individuals. Variable

shedding in individuals is common in sewage-associated MST markers such as the HF183 marker (69), and person-to-person variability for the gut microbiome has been well documented in the literature (70-72). Variability in culturable EcH8 performance could occur across geographical regions, and parameters such as sensitivity and specificity should be evaluated in new study locations with local fecal/untreated sewage samples. Recycled water applications and usage vary widely across the U.S. and in other countries, affecting the potential of recycled water to confound surface water quality testing, therefore knowledge of irrigation and other practices that may deliver recycled water or treated wastewater to surface waters is necessary to maximize interpretation of MST results.

Future directions for the culturable EcH8 method of detecting viable sewage-associated *E. coli* should explore strategies to increase the number of *E. coli* isolates tested. Testing more *E. coli* colonies will improve method sensitivity, while extracting DNA from composite samples made from multiple colonies can improve workflow and adoptability of this approach. Coupled with a standard MST marker that targets untreated sewage, e.g., HF183 and culturable EcH8 can be used as a confirmation step for the identification of untreated sewage contamination in surface waters and will improve MST interpretations in water bodies that receive substantial inputs of recycled water.

In summary, this study showed that DNA in recycled water released from an AWT facility could raise HF183 levels 1000-fold in receiving waters. We demonstrated the persistence of *E. coli* and MST marker DNA through AWT and CWT in six WWTFs, supporting the premise that DNA released in recycled water and other disinfected effluent can cause a false-positive indication of untreated sewage contamination in environmental waters. The comparison of DNA removal through wastewater treatment in facilities with different levels of treatment showed that AWT

facilities were more effective than CWT facilities at removing DNA, particularly in the case of crAssphage CPQ_056. We demonstrated that culturable EcH8 has strong potential to discriminate between the presence of untreated sewage compared to disinfected effluent in environmental waters by demonstrating undetectable levels of cultured *E. coli* in recycled water, and thus the absence of EcH8 in culturable *E. coli* isolates, and showing that culturable EcH8 can be detected in contaminated surface waters, and that its detection is correlated with HF183 concentrations. The usefulness of culturable EcH8 to discriminate untreated sewage from recycled water sources can be improved by modifying the method to screen more *E. coli* colonies per sample, thus increasing sensitivity. It should also be further explored by measuring the persistence of culturable EcH8 through varying wastewater treatment systems and how it persists in surface waters exposed to environmental conditions.

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Table 2.1. Characteristics of advanced (AWT) and conventional (CWT) wastewater treatment facilities. All untreated sewage influent samples were collected before treatment, while all recycled water samples were collected post-disinfection, and prior to entering the distribution system.

	WWTF Capacity (MGD)	Recycled Water Produced (MGD)	Estimated Population Served	Treatment Processes	Disinfection
Conventional					
A	20	16.4	126,880	Primary/secondary Activated sludge treatment	sodium hypochlorite
B	20	10	80,560	Primary/secondary Activated sludge treatment	sodium hypochlorite
C	16	8	62,320	Primary/secondary Activated sludge treatment	sodium hypochlorite
Advanced					
D	12	9.1	172,346	1) Primary/secondary 2) Anoxic basins and oxidation ditches 3) Dual media deep-bed denitrification filters	UV
E	33	14.6	200,000	1) Primary/secondary 2) Activated sludge treatment in an anoxic/aerobic configuration 3) Deep-bed denitrification filters with methanol addition	chlorine and sodium hypochlorite
F	9	6.5	100,000	1) Primary/secondary 2) Five-stage Bardenpho activated sludge process	chlorine

Table 2.2. HF183 and culturable *E. coli* concentrations (log_{10} GC or CFU/100 mL) in a stream that receives recycled water from an AWT facility. The stream was sampled upstream of the outfall, at the outfall, and downstream before, during and after discharge of recycled water.

Temporal relationship to discharge	**Upstream Site** log_{10} CFU or GC/100 mL		**Recycled Water Discharge Outfall** log_{10} CFU or GC/100 mL		**Downstream Site** log_{10} CFU or GC/100 mL	
	E. coli	HF183	*E. coli*	HF183	*E. coli*	HF183
Prior to discharge	2.88	< LOD[a]	2.54	1.30	2.30	< LOD
During discharge	2.00	< LOD	< LOD	4.15	3.31	3.58
After discharge[b]	1.88	< LOD	1.95	< LOD	2.43	1.63

[a] < LOD = below qPCR limit of detection.

[b] Sampled 23 hours after discharge was discontinued.

Table 2.3. Statistical comparisons of microbial variables measured by qPCR in recycled water produced in AWT and CWT facilities. Data are expressed as concentration (log_{10} GC/100 mL), log_{10} reduction, and frequency of detection. Data from like facilities (AWT or CWT) were pooled (n=9). Variables were individually compared between AWT and CWT facilities by Kruskal-Wallis[a] rank sum tests and compared as a group by permutational multivariate analysis of variance[b]. Differences in frequency of detection were compared by Fisher's Exact test[c]. P-values < 0.05 are bolded.

Univariate qPCR Variables	**P value: Differences Among Median Log_{10} Concentrations**	**P value: Difference among Log_{10} Reduction**	**P Value: Difference in Frequency of Detection**
EC23S857[ac]	0.7573	0.2004	1.0000
HF183[ac]	0.1957	0.2332	**0.0294**
H8[ac]	**0.0218**	0.1451	**0.0091**
CPQ_056[ac]	**0.0003**	**0.0004**	0.0824
Multivariate All qPCR Variables[bc] (advanced versus conventional)	**0.0120**	**0.0110**	**< 0.0001**

Table 2.4. Frequency of detection of microbial variables (general fecal and sewage-associated MST markers) measured by qPCR in recycled water from advanced (AWT) and conventional (CWT) wastewater treatment plants. Each plant was sampled on three separate events.

	EC23S857	**HF183**	**H8**	**CPQ_056**
Conventional	**Frequency of detection (%)**			
A	100	100	100	100
B	100	100	100	100
C	100	100	100	100
CWT data combined (n=9)	100	100	100	100
Advanced				
D	100	100	100	100
E	100	33	0	0
F	100	0	0	67
AWT data combined (n=9)	100	44	33	56
AWT and CWT data pooled (n = 18)	100	72	67	78

Table 2.5. Mean culturable *E. coli* concentrations (log_{10} CFU/100 ml) (± std. error) and the proportion of tested colonies positive for the H8 gene (culturable EcH8) in untreated sewage from AWT and CWT plants. Each plant was sampled on three separate events.

Conventional WWTF Untreated Sewage	**Culturable *E. coli* (log_{10} CFU 100 ml^{-1})**	**Colonies tested for EcH8**	**Proportion EcH8-positive (%)**
A	6.67 ± 0.04	90	17.7 ± 3.9
B	6.59 ± 0.04	90	15.7 ± 1.3
C	6.52 ± 0.12	90	15.7 ± 4.3
Advanced WWTF Sewage			
D	6.56 ± 0.05	90	9.0 ± 2.0
E	6.46 ± 0.03	90	8.0 ± 1.0
F	6.47 ± 0.03	90	17.0 ± 5.8

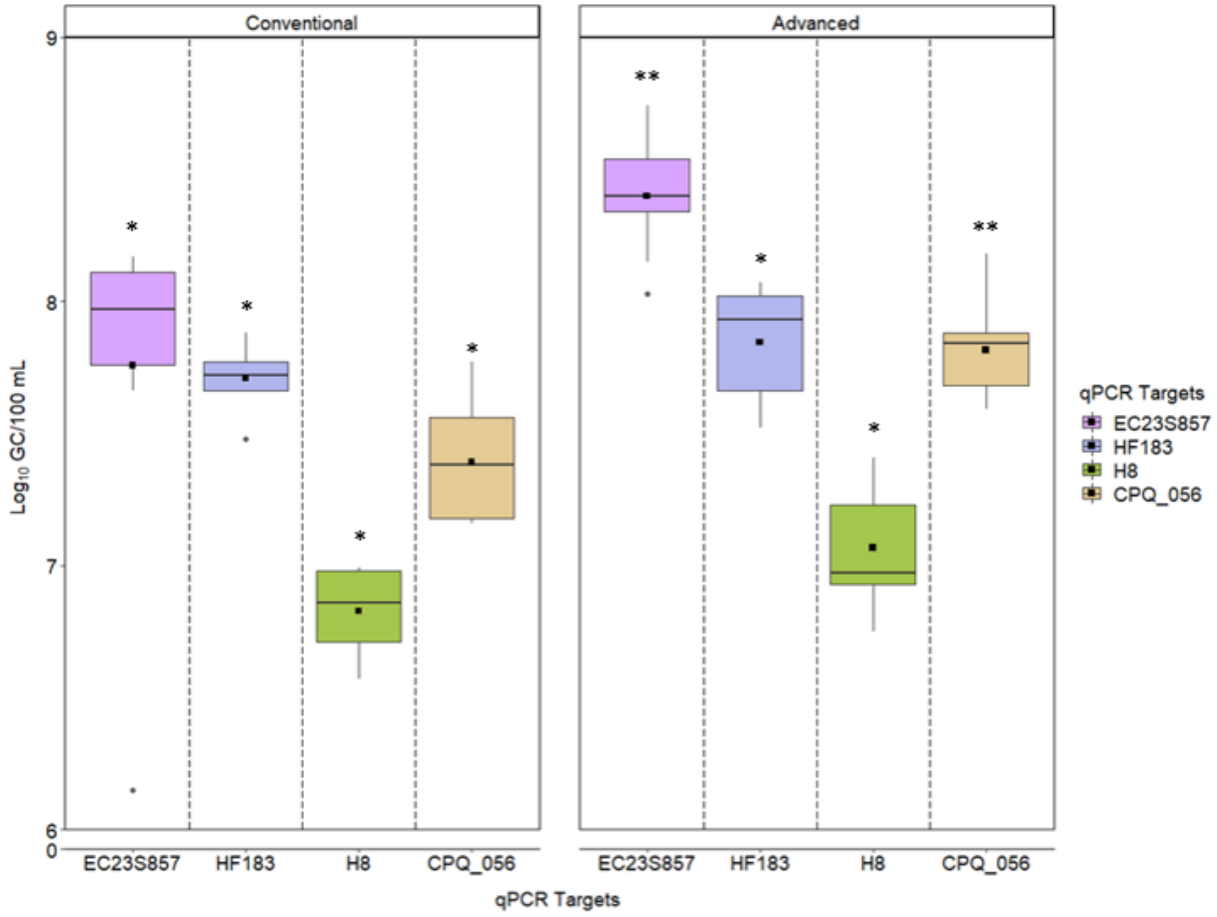

Figure 2.1. Concentrations of microbial variables (log_{10} GC/100 ml) in untreated sewage from advanced (AWT) and conventional (CWT) facilities. The interquartile range (25^{th} and 75^{th} percentile) includes the log_{10} medians (black horizontal bar) and means (black square) for each qPCR marker across conventional and advanced WWTFs. Boxplot whiskers represent the 10^{th} and 90^{th} percentile values. Outliers are displayed as black dots above or below each boxplot, the y-axis was truncated to show data ranging from 6.0 to 9.0 log_{10} GC/100 mL. Each plant was sampled on three separate events. Asterisks denote comparison of values between conventional and advanced WWTFs. Variables with different numbers of asterisks across the WWTF types are significantly different ($\alpha = 0.05$), e.g., EC23S857 is significantly greater in AWT compared to CWT facilities.

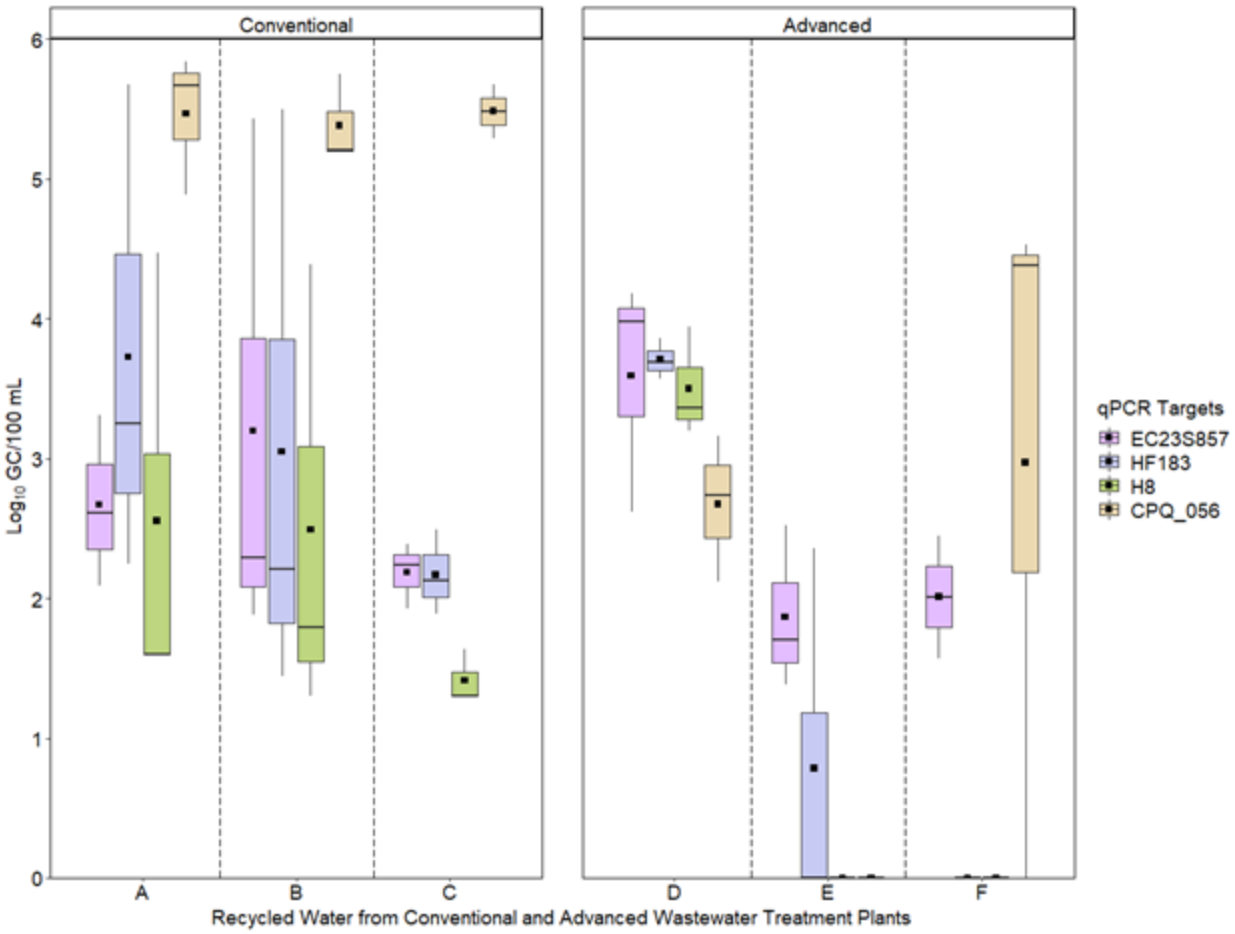

Figure 2.2. Concentrations of microbial variables (log_{10} GC/100 ml) in recycled water from AWT and CWT facilities. The interquartile range *(*25^{th} and 75^{th} percentile*)* includes the log_{10} medians (black horizontal bar) and means (black square) for each marker across conventional and advanced WWTFs. Boxplot whiskers represent the 10^{th} and 90^{th} percentile values. Each plant was sampled on three separate events.

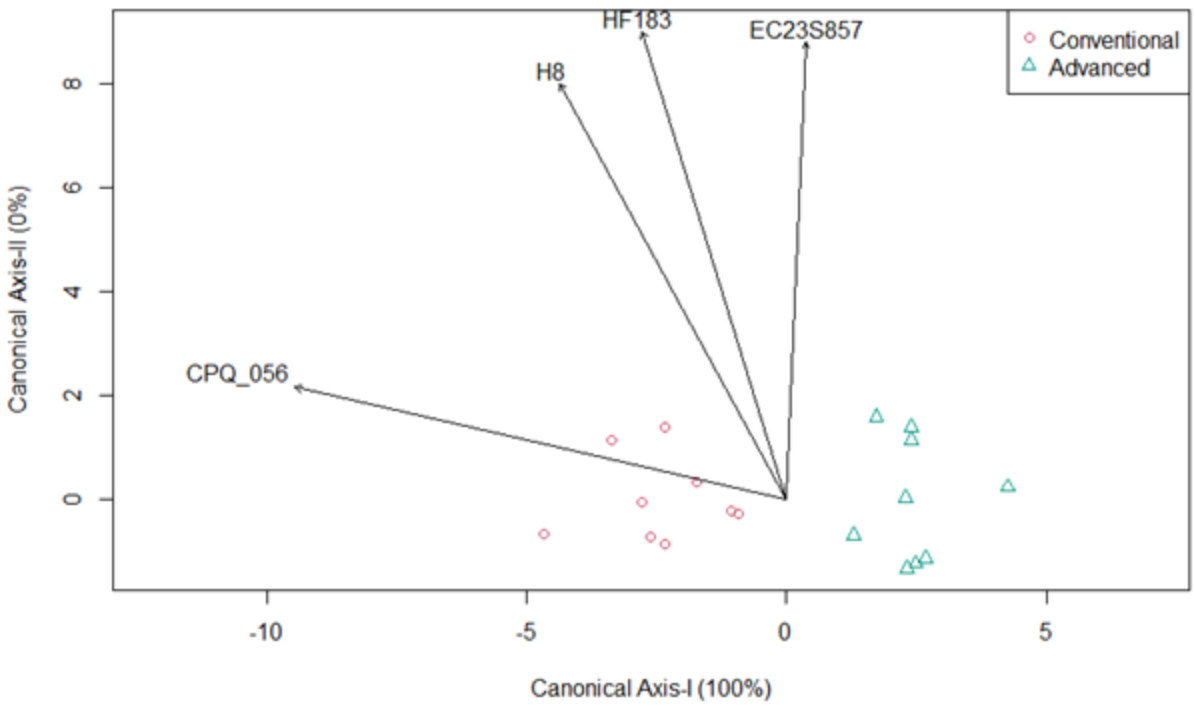

Figure 2.3. Relationships among microbial variables measured by qPCR in recycled water produced by AWT and CWT facilities (conventional = red circles, and advanced = green triangles) analyzed by canonical analysis of principal coordinates and linear discriminant analysis. Canonical axis I (horizontal) explained 100% of the variability, while canonical axis II (vertical) explained 0% of the variability observed.

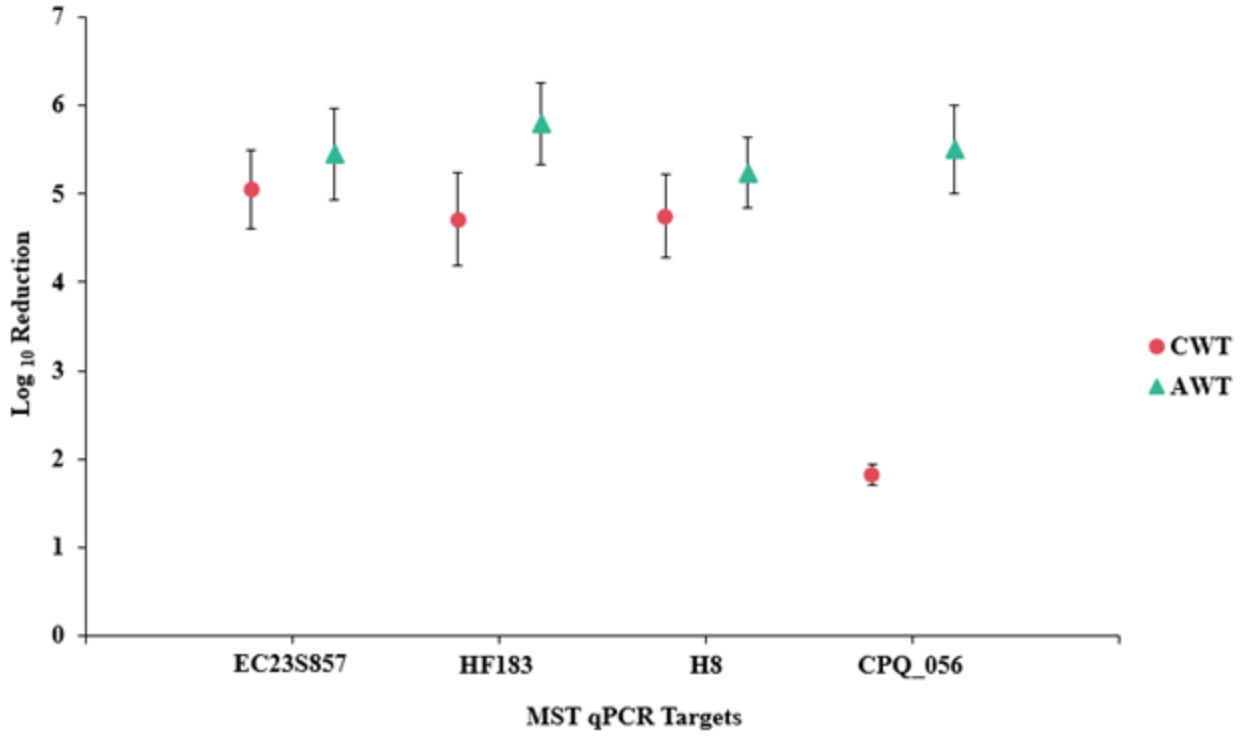

Figure 2.4. Mean log_{10} reduction (decay) of microbial variables measured by qPCR in recycled water from AWT and CWT wastewater treatment facilities. Error bars are standard error of mean log_{10} reduction values.

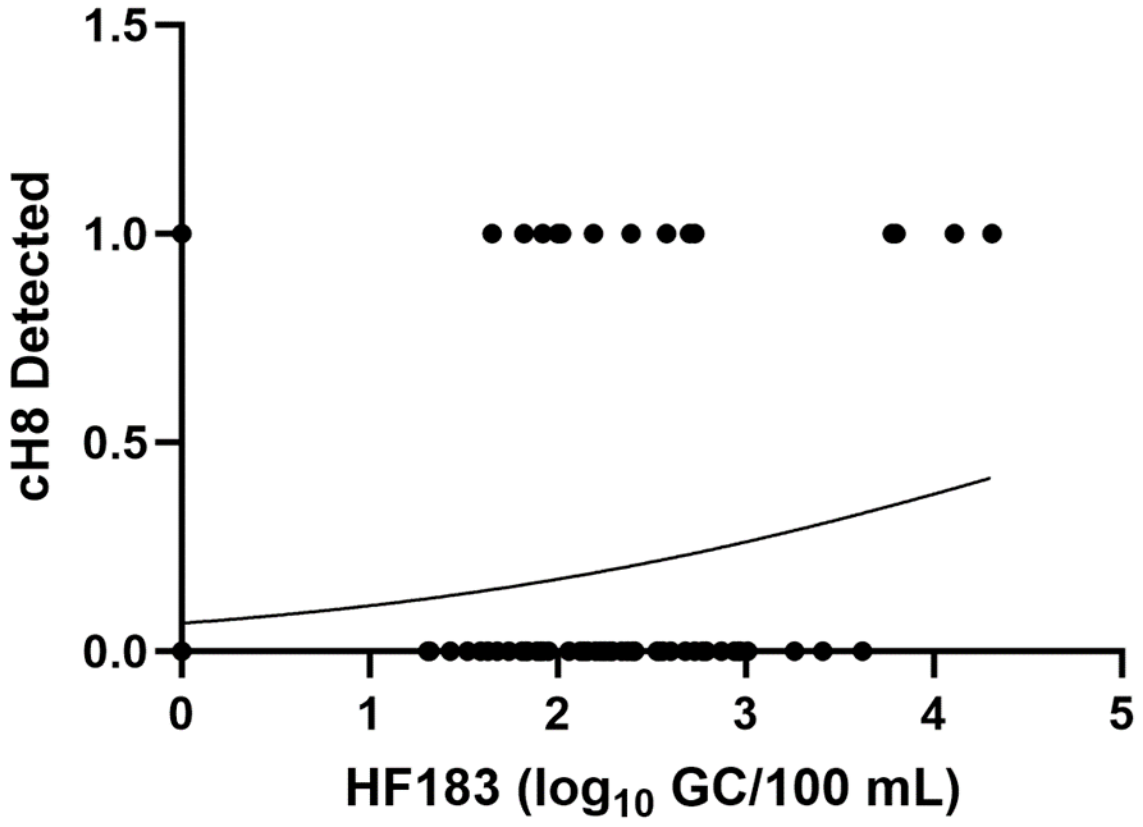

Figure 2.5. Binary logistic regression of a significant positive relationship between culturable EcH8 detection and HF183 concentrations (log_{10} GC/100 mL) in contaminated surface water samples (n=103).

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CHAPTER THREE: MICROBIAL SOURCE TRACKING MARKER DNA FROM UNTREATED SEWAGE DECAYS MORE RAPIDLY IN FRESHWATER THAN DNA FROM RECYCLED WATER

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Abstract

Sewage-associated HF183 and CPQ_056 are used globally to track sewage pollution in surface waters by qPCR; however, both DNA markers can persist through wastewater treatment and remain quantifiable even in highly treated recycled water, which seldom contains detectable levels of culturable *Escherichia coli*. Persistent DNA in recycled water can confound interpretation of microbial source tracking (MST) efforts to assess sewage contamination; however, culturable *E. coli* with the sewage-associated H8 gene (EcH8) can be used to discriminate between sewage and recycled water. Persistence of the qPCR signal of MST marker DNA from recycled water is underexplored, and little is known about the persistence of EcH8 in surface waters. This study compared decay of DNA markers EC23S857 (*E. coli*), HF183, and CPQ_056 in river water spiked with (i) recycled water or (ii) untreated sewage, and the environmental persistence of EcH8 from sewage. Sewage or recycled water was spiked into river

water in shaded outdoor freshwater mesocosms sampled over five days. Decay of qPCR variables assessed by multivariate analysis was significantly more rapid in the untreated sewage treatment compared to the recycled water treatment, and light intensity contributed significantly to decay. Culturable EcH8 remained detectable for five days in the sewage treatment but was undetectable in recycled water. On day 5, median $\log_{10}$ reduction values (LRV) of microbial variables measured by qPCR in recycled water treatments were 0.61 (EC23S857), 0.68 (HF183), and 0.44 (CPQ_056). LRV of microbial variables in with the sewage treatment were 0.39 (EC23S857), 2.83 (HF183), and 1.0 (CPQ_056), indicating comparatively greater rate of decay of HF183 and CPQ_056 in sewage compared to recycled water. EC23S857 decayed significantly more slowly than HF183 or CPQ_056 in the sewage treatment, but differences were not significant in the recycled water treatment. These data provide a deeper understanding of the extended environmental persistence of DNA from recycled water and support the usefulness of culturable EcH8 for detecting untreated sewage in recreational waters that are also impacted by recycled water and other disinfected discharges of wastewater.

Importance

Microbial source tracking (MST) in environmental waters can discriminate sewage from animal fecal sources with sewage-associated genes measured by qPCR. However, DNA in untreated sewage can persist through wastewater treatment and enter surface waters through release of treated sewage from permitted discharges and as recycled water. Differentiating recycled water from sewage in surface waters is necessary for accurate health risk assessments due to the greater potential for the presence of infectious pathogen presence in sewage vs recycled water. Furthermore, the ability to identify untreated sewage will improve pollution mitigation efforts and avoid unnecessary investigations due to the qPCR signal from treated discharges. In this

study, we demonstrate the environmental persistence of MST markers from two human sources (recycled water and untreated sewage) and apply the culturable EcH8 method with viable *Escherichia coli* to specifically identify untreated sewage in river water over time.

Introduction

Microbial source tracking (MST) methods are frequently adopted by local and state agencies to measure sewage-associated DNA markers such as HF183 in *Bacteroides dorei* (1), and CPQ_056 carried by crAssphage viruses (2). HF183 and CPQ_056 concentrations are reported in multiple studies that investigate the impact of sewage pollution on environmental waters (3-6). However, MST generally involves qPCR methods that lack the ability to distinguish viable microorganisms from nonviable cells and free DNA, as qPCR measures DNA. This is an issue since MST markers such as HF183 and CPQ_056 can persist through wastewater treatment and remain quantifiable in disinfected effluent and recycled water at levels sufficient to increase HF183 levels several orders of magnitude in a freshwater body (4, 7-11). This phenomenon is also an issue in measurements of general fecal pollution for recreational water quality assessment because DNA markers of FIB such as EC23S857 can also persist through wastewater treatment (11). To our knowledge, no studies have previously examined the persistence of DNA markers in recycled water once it is introduced to an environmental matrix, and how it compares to the sewage qPCR signal under similar conditions. Free nucleic acid from recycled water could be more susceptible to degradation from abiotic or biotic pressures and may have a greater rate of decay compared to nucleic acids in intact cells from untreated sewage.

One challenge for recreational water quality studies is that disinfected effluent and recycled water are sources of DNA originally derived from sewage. The source of the microbial DNA can be incorrectly identified as untreated sewage, which can lead to an overestimation of health risk.

Untreated sewage pollution is a greater public health risk than recycled water due to the presence of a diversity of viable pathogens and human specific viruses in sewage (12-15), while recycled water poses a lower health risk due to low levels of viable microorganisms (16-20). The H8 marker gene in culturable sewage-associated *E. coli* (EcH8), is a sensitive and specific marker for sewage (21-23) and is one possible solution for discriminating sewage from recycled water, as this viable target is present only in sewage (11). However, the persistence of EcH8 in environmental waters compared to all *E. coli* and MST markers has not been explored. This approach to detecting sewage will not be very useful to MST efforts if EcH8 die rapidly in aquatic environments, or if EcH8 is persistent and survives longer than generic *E. coli,* MST markers, and pathogens in environmental waters.

Multiple environmental factors can influence decay of microbial variables, but factors such as sunlight that has a known effect on decay of microbial variables from untreated sewage should also be examined on how it could impact decay of culturable EcH8 and DNA markers from recycled water. Sunlight is known to be a major contributing factor to the decay of bacteria in surface waters and can have differential impacts on DNA markers from sewage (reviewed in 24). Concentrations of culturable *E. coli* from sewage can be reduced by more than one hundred-fold in environments exposed to direct sunlight (25-27). Previous studies of microbial decay in untreated sewage have found that MST markers (i.e. HF183 and CPQ_056) persist and remain quantifiable after a week in environmental conditions, even in cases when exposed to direct sunlight (25, 28-31). The proportion of culturable EcH8 in total *E. coli* from sewage ranged from 8 to 20% in U.S. studies (11, 21). If decay, measured as loss of culturability, in surface waters occurs rapidly for EcH8 compared to DNA markers, then this method may only be useful for detecting recent sewage contamination events. Published studies generally contrast full sun with

complete shade (frequently termed a dark treatment) (e.g., 27, 32-34) and little information on the influence of intermediate shade, such as one might find in streams covered by tree canopy, is available. Furthermore, the current literature contains no examples for the relationship between sunlight and decay of DNA markers from recycled water, which will be important to understand as recycled water use increases along with its possible interference in data interpretation for recreational water quality analyses.

The objectives of this study were (i) to compare the decay of DNA markers of sewage from recycled water or untreated sewage in a shaded freshwater environment, and (ii) to compare the decay rate of culturable EcH8 to that of the DNA markers. We tested two major hypotheses in this study: (i) DNA markers from recycled water, which are derived from mainly from dead cells and extracellular DNA, will decay more rapidly in river water compared to DNA from untreated sewage, which is derived from a mixture of live cells, dead cells, and extracellular DNA and (ii) the persistence of culturable EcH8 in the water mesocosms will be comparable to that of the DNA markers.

Methods

Site Description and Freshwater Collection

Water was collected from the same site (28.088007, -82.348996) in the Hillsborough River in Tampa, FL during the morning on day 0 of each trial (n = 3). The site is upstream of urban Tampa. Two hundred L of river water was collected per trial in sterile 20 L carboys and transported to a shaded research site at the University of South Florida (USF) Botanical Gardens (28.057593, -82.425086) to fill outdoor mesocosms. Remaining water was transported to the laboratory for analysis of background levels of microorganisms, and to prepare treatment spikes for 5-day mesocosm experiments.

Mesocosm Inocula

Recycled wastewater and untreated sewage influent were collected from a conventional wastewater treatment facility in St. Petersburg, Florida on three separate dates (April – May 2023). This facility serves approximately 126,880 residents and produces about 16.4 million gallons per day of recycled water. Wastewater in this facility is subjected to primary and secondary treatment by an activated sludge process, and effluent is disinfected with sodium hypochlorite before entering the recycled water distribution system. Recycled water (1.5 L) and untreated sewage (1.5 L) were collected in sterile 2 L polypropylene containers and transported at 4 °C on wet ice to the laboratory. Dilutions were prepared in two separate sterile 10 L carboys by adding 600 mL of recycled water or untreated sewage into 5,400 mL of river water to reach a 10 % solution of each inoculum. A sterile magnetic stir bar was used to mix each sample for 1 minute before preparing dialysis bags.

Outdoor Mesocosm Preparation and Physical Water Property Measurements

Mesocosms consisted of Sterilite plastic storage boxes (86 cm length × 47.6 cm width × 33 cm height) with fishing line (25-pound test line) and stainless-steel heavy-duty metal clamps. Mesocosms were cleaned with 70% alcohol and flushed with ambient river water prior to filling with river water to create a reservoir. Dialysis tubing cellulose sample bags (75 mm flat width, 13–14 kD pore size; molecular weight cut-off, Spectrum Labs, Rancho Dominguez, CA) were used following previous studies (27, 31, 35); one study demonstrated that dialysis tubing allowed low attenuation (< 10%) of sunlight (32). Dialysis bags contained 200 mL of each treatment (10 % recycled water or untreated sewage, see details below) and were placed approximately 5 – 10 cm below the water surface in each mesocosm. Each bag was tied to approximately 40 g of swivel fishing weights at the bottom to keep them in a vertical orientation in mesocosms. River

water was continuously circulated in each mesocosm with an aquarium pump throughout the entire experiment. Mesocosms were topped off with additional river water each day to account for evaporation loss. Ambient water temperature and light intensity conditions were measured in each mesocosm on an hourly basis using Onset HOBO MX2202 Waterproof Bluetooth Pendant light and temperature data loggers (Onset Computer Corporation Bourne, MA) in dialysis bags approximately 5 cm below the water surface.

Thirty dialysis bags for each pollution type (recycled water or untreated sewage) were incubated in outdoor mesocosms for up to five days. Five dialysis bags from each treatment were harvested at the beginning of the experiment (day 0) and every 24 hours up to day 5. Mesocosm experiments were repeated on three separate trials (Figure C.1). Harvested dialysis bags were transported with 100 mL of ambient river water in sealed plastic bags on wet ice to the laboratory. The contents of each dialysis bag were separated into 180 mL for DNA extraction and 20 mL for culture of *E. coli.* Samples were processed within one hour of harvesting.

Nucleic Acid Purification

Forty-five - 180 mL of sample from each dialysis bag was filtered onto a mixed cellulose ester filter (47 mm diameter 0.45 µm pore size; Fisherbrand) until refusal. Controls included five technical replicates for background levels of microorganisms in 200 mL of river water, 20 mL of recycled water, or 1 mL of untreated sewage that was filtered as described above for each trial. The volume filtered was recorded and used to normalize values to gene copies/100 mL. DNA was extracted by processing membranes in a Dneasy PowerWater™ DNA extraction kit (Qiagen) for qPCR analysis following manufacturer's instructions. Nucleic acids were eluted in 100 µL elution buffer for river water, sewage and samples spiked with sewage, and in 50 µL

buffer for recycled water and samples spiked with recycled water. DNA was stored up to 120 hours at -20 °C until qPCR amplification was conducted.

QPCR Amplification

Quantitative PCR assays included the general *E. coli* target EC23S857 (36), and sewage-associated HF183 (1) and crAssphage CPQ_056 (2). Primer and probe concentrations/sequences and qPCR assay conditions are reported in supplementary material (Table C.1) for each assay. QPCR amplifications was conducted in 25 µL reactions in duplicate using 12.5 µL TaqMan Environmental Master Mix 2.0 (Applied Biosystems) and 5 µL of template DNA per reaction in a Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories; California, US). Standard curves were constructed from gene fragments in gBlocks (Table C.2) containing the target sequences, and reference DNA material (internal amplification control) was included for each sample to test for inhibition according to guidelines in USEPA method 1696 (1).

All standard curves ranged from 5 to 10^7 gene copies (GC) per reaction and performance metrics included efficiencies between 90 and 110%, and > 0.98 R^2 values. In sewage-spiked mesocosms, the limit of detection (LOD) was 50 GC/100 mL and the limit of quantification (LOQ) was 100 GC/100 mL for each DNA marker. In mesocosms spiked with recycled water, the LOD was 25 GC/100 mL and LOQ was 50 GC/100 mL for each DNA marker. Inhibition of qPCR amplification was not detected in any of the samples tested in this study. Negative controls for each instrument run included 6 lab blanks (phosphate buffered saline, pH 7.5), 6 extraction blanks, and 6 non-template controls (nuclease free water used in qPCR) which were all negative for each qPCR target in this study. All sewage-associated markers in Hillsborough River were below the LOD prior to inoculation of mesocosms.

Culture of E. coli and Molecular Detection of H8

Bacteria from dialysis bags were concentrated on sterile mixed cellulose ester filters (47 mm diameter 0.45 µm pore size; Fisherbrand). Colony forming units (CFU) of *E. coli* were enumerated on membrane thermotolerant *Escherichia coli* (mTEC) agar plates according to USEPA method 1603 (37). For sewage-spiked mesocosms, samples were diluted 10^{-3} from day 0 to day 3 and 10^{-2} to 10^{-1} for both day 4 and day 5, and 1 mL was filtered in duplicate from each dilution. In mesocosms spiked with recycled water, 10 mL was filtered in duplicate from each dialysis bag per day. The LOD for culturable *E. coli* in this study was 10^{3} CFU/100 mL for untreated sewage spiked samples, and 10^{1} CFU/100 mL for recycled water spiked samples. Well-separated colonies with characteristic *E. coli* morphology and coloration were individually picked from mTEC plates (30 isolates per dialysis bag whenever possible) and composited in 50 µL of reagent grade nuclease-free water in a 100 µL microcentrifuge tube for H8 (culturable EcH8) analysis. Each colony composite was incubated for 10 min at 100 °C in a thermal cycler as described previously (21).

Confirmation of *E. coli* was conducted through real-time PCR amplification of an *E. coli* specific β-glucuronidase *uid*A gene (38) and for presence of the H8 gene by real-time PCR. PCR amplification was conducted in 25 µL reactions that included 12.5 µL TaqMan Environmental Master Mix 2.0 (Applied Biosystems) and 5 µL of template DNA per reaction in a Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories; California, US). Primer and probe concentrations/sequences and PCR assay conditions are reported in supplementary material (Table C.1). A positive control for *uid*A and H8 consisting of genomic DNA from *E. coli* ATCC 13706 was included in each run. A H8 negative control (*E. coli* ATCC 11775) and a no template control (nuclease free water) was also included in each run.

Data Analyses

Statistical analyses in this study were executed in GraphPad Prism version 10.2.3 and R version 4.1.3 (39), and included descriptive statistics, hypothesis testing, and correlation analyses. All qPCR data were log_{10}-transformed prior to statistical analysis. A significance threshold of $\alpha = 0.05$ was set for all statistical tests. LOQ was defined as the lowest standard concentration that consistently amplified in all three replicates, while LOD was the lowest standard concentration in which at least 2 out of 3 replicates consistently amplified. Data that were below the LOQ were observed only in samples from the recycled water treatment and were considered censored. u-scores were calculated based on v 1.2 of u-score script written by D. Helsel (available in PracticalStats.com) (40). Each observation in each data set was assigned a u-score based on rank, which were utilized to compute statistical analyses that tested relationships between microbial variables and differences by treatment type. U-scores of censored data were expressed as a range from 0 to LOD - 1 for observations below the detection limit and LOD to LOQ - 1 for observations that were detected but not quantifiable.

Descriptive statistics (median and quartiles) were calculated by treatment type (recycled water or untreated sewage) for culturable *E. coli* and DNA markers (EC23S857, HF183 and CPQ_056) through the R package stats (version 4.1.3). Log_{10} reduction values were calculated by subtracting the log_{10} concentration of the mean on day-5 from the mean log_{10} concentration on day-0 for each microbial variable. Multivariate analysis on log_{10} reductions of all DNA markers was done with each dialysis bag treated as a replicate to determine the effect of source (recycled water or untreated sewage) and environmental variables on decay. Multivariate statistics were executed by PERMANOVA though the package RVAideMemoire (version 0.9-83-3), and redundancy analysis was conducted with the package BiodiversityR (version 2.15-4).

In univariate analyses, LRVs from recycled water or untreated sewage treatment data were calculated from five replicate dialysis bags per marker, per trial. A two-way ANOVA was executed to test the effect of factors (i.e., trials and DNA markers) on differences in decay in recycled water and sewage treatments separately. The Tukey HSD post hoc test was conducted for multiple pairwise comparisons among trials and DNA markers. Differences in the frequency of detection for culturable EcH8 across trials were determined with the Fisher exact test (41) and the R package rstatix (version 0.7.2), with Bonferroni method for p-value correction. Relationships among $\log_{10}$ reductions of microbial variables for each source type was determined with Kendall's rank correlation tau, where the coefficient (tau) can range from -1.0 to 1.0. A value of -1.0 designates a perfect negative correlation in rank between two variables; a value of 1.0 indicates a perfect positive correlation. A value of 0 demonstrates that no relationship in rank exists between two variables.

The univariate analysis for differential decay of microbial variables between recycled water and sewage treatments included each experimental trial as a biological replicate, providing three mean $\log_{10}$ reduction values for each DNA marker in the recycled water or sewage treatments. A linear mixed effect model was constructed for each marker which included a fixed effect ($\log_{10}$reductions) and one random effect (trials). Models were tested by ANOVA and were assembled with the method REML using the R package nlme (version 3.1-155). Log-linear regression models were ensembled with the tool GinaFiT (42) for each microbial variable in data from recycled water or untreated sewage-spiked mesocosms across three trials. Fit of each model was assessed by comparing adjusted R^2 and root mean square error (RMSE) provided by the GinaFiT tool. The first-order decay rates (k) in $\log_{10}$/day were calculated by dividing concentrations of day 5 (C_t) by day 0 (C_0) and transforming by natural log then dividing by time

(T) which was 5 days in this study as shown in equation (i) (43). T_{90} values were defined as the time (days) for one-log (90%) reduction and were calculated using equation (ii) (43).

$$\ln(C_t/C_0) \,/\, T = -\, k \qquad \text{(i)}$$

$$T_{90} = -\ln(0.1) \,/\, k \qquad \text{(ii)}$$

Results

Prior to inoculating river water with recycled water or sewage, concentrations of each microbial variable were measured in river water, recycled water, and untreated sewage for each experimental trial (Table C.3). In river water, the highest mean concentrations of culturable *E. coli* and EC23S857 were 1.89 and 2.82 CFU or GC/100 mL, respectively, while sewage-associated markers were below the LOD in each trial. In recycled water, culturable *E. coli* were below the LOD in each trial but the mean of DNA markers ranged from ~ 4 to 5 $\log_{10}$ GC/100 mL. In untreated sewage, all microbial variables were detected and means ranged ~ 6 to 8 $\log_{10}$ CFU or GC/100 mL. In this study, water temperature means ranged 21.3 – 23.2 °C while the average for light intensity ranged 111 - 155 lum/ft.^{2} across the three trials (Table C.4). No significant differences were found among trials for both temperature and light measurements.

Comparison of Decay ($\log_{10}$ reduction) of DNA Markers Measured by qPCR from Recycled Water vs Sewage Treatments

The decay of DNA markers in river water spiked with recycled water or untreated sewage was analyzed on three dates (trials) conducted in March, April, and May 2023 with the objective of determining which factors (i.e., markers and source) contributed to $\log_{10}$ reductions. Multivariate redundancy analysis was executed to explore decay differences between sources and to examine relationships between decay and environmental variables. Decay of DNA markers was significantly greater ($p = 0.001$) in river water spiked with untreated sewage (sewage treatment)

compared to recycled water-spiked samples (recycled water treatment); however, the model only explained 14% of the variability in the data (Figure 3.1). Water temperature and light intensity were included as explanatory variables in the multivariate model, where only light ($p = 0.033$) was a significant factor in decay of DNA markers and not temperature ($p = 0.265$).

The effect of DNA source and light intensity on decay was further explored by univariate analyses following the significant findings in the multivariate model. In linear mixed effect models analyzed by ANOVA, source (recycled water vs sewage treatment) did not have a significant effect on the LRV of any DNA marker (EC23S857, HF183, or CPQ_056) (Figure 3.2, Table 3.2). Decay of EC23S857 and CPQ_056 in the sewage treatment were significantly and positively correlated with visible light measurements, while an α equal to 0.10 would have resulted in a positive significant relationship for HF183 ($p = 0.0645$, $\tau = 0.394$) (Table C.5).

Alternative metrics for decay (i.e., decay rate *k*, and T_{90}) of DNA markers were examined to facilitate comparisons with measurements reported in the literature. The other decay metrics we calculated tracked LRV trends, as expected. For example, first-order decay constants (*k*) for CPQ_056 and HF183 in the sewage treatment ranged from 0.27/day to 1.37/day across three trials, which was greater compared to the recycled water treatment with *k* values ranging from 0.14/day to 0.64/day (Table 3.3), respectively. The time for one $\log_{10}$ reduction (T_{90}) of HF183 and CPQ_056 were generally greater in recycled water treatments compared to sewage treatments where the T_{90} ranged from 1.7 to 8.6 days across the three trials (Table 3.3).

Effect of Trials and DNA Marker on Decay (log_{10} reduction) in Recycled Water or Sewage Treatments

Decay rates (LRV) between trials was compared among DNA markers separately in the recycled water treatment and sewage treatment. A two-way ANOVA was executed to determine the effect

of factors (i.e., trials and markers) on decay of DNA markers for each treatment. In the recycled water treatment, trials was a significant factor in decay (Table 3.1, Table C.6). DNA markers in the recycled water treatment in Trial 1 experienced the least decay (Figure 3.3). DNA marker was not a significant factor in decay, and there was no significant interaction between trials and DNA markers, although HF183 LRV was significantly different between trial 1 and 3 (Table C.6). On day 5, median HF183 LRVs across trials were greatest (0.40 – 1.71) followed by EC23S857 (0.44 – 1.67) and finally crAssphage CPQ_056 (0.23 – 0.57) (Figure 3.3). In the sewage treatment, trials, markers and the interaction between the two factors had a significant effect on LRV differences (Table 3.1). On day 5, the range of median LRV across trials for HF183 (0.68 – 2.96) was greatest followed by CPQ_056 (0.50 – 1.05) and EC23S857 (- 0.50 – 0.39) (Figure 3.3). The LRVs of each DNA marker were significantly different from each other in all trials except for HF183 and CPQ_056 in Trial 1 (Table C.7).

Culturable E. coli and EcH8 Decay in Mesocosms

Decay of culturable *E. coli* and culturable EcH8 was assessed to contrast with the DNA markers. Decay of culturable EcH8 is expressed as frequency of detection, as composite samples containing multiple colonies were tested from each dialysis bag on a presence/absence basis. *E. coli* were never detected in recycled water and were infrequently detected (24 – 32%) across trials in the recycled water treatment, where levels were consistent with those observed in unspiked river water. We therefore considered these *E. coli* to be contributed from the river rather than from recycled water, and data from the recycled water treatment was excluded from statistical analyses.

In the sewage treatment, the median LRV on day 5 for culturable *E. coli* was - 0.41, 0.40, and 1.07 for Trials 1, 2, and 3 respectively. The one-way ANOVA showed that trials was a

significant factor ($p < 0.0001$) for LRV differences in culturable *E. coli* ($p < 0.0001$), where each trial was significantly different from the other ($p < 0.0001$). In the sewage treatment, the frequency of detection for culturable EcH8 was 100% on day 0 for all three trials and ranged from 40 to 60% after 5 days exposed to environmental conditions. Culturable EcH8 frequency of detection was not significantly different ($p > 0.9999$) across the three trials.

Correlations Among Microbial Variables in Recycled Water or Sewage Treatments

In recycled water treatments, a significant positive correlation was observed between HF183 and EC23S857 LRVs, but neither marker was correlated with CPQ_056 decay (Table C.8, Figure C.4). The LRVs of all variables, including culturable *E. coli* and DNA markers, were positively correlated in the sewage treatment (Table C.8, Figure C.5).

Discussion

Quantification of DNA markers such as HF183 and CPQ_056 by qPCR is a widely accepted strategy to identify untreated sewage in surface waters; however, multiple studies have found that these DNA markers can persist through wastewater treatment and complicate MST efforts when recycled water or treated effluents enter surface waters (4, 7-11). To our knowledge, this is the first study to explore the persistence of DNA markers from recycled water compared to markers from sewage under environmentally-relevant conditions.

Direct discharge of disinfected effluent or excess recycled water into a surface water body is a common occurrence in the U.S. Many receiving waters have a high percentage of canopy cover which results in shaded conditions. For example, Turkey Creek in Hillsborough County, Florida receives excess recycled water discharged from an advanced wastewater treatment facility. The stream is moderately shaded with a mean canopy cover of 63.5% (44). We showed that discharge of recycled water increased concentrations of HF183 by three orders of magnitude in Turkey

Creek (11). Excess recycled water can also be injected into aquifers, where there is no sunlight exposure and microbial population is lower compared to surface waters, for storage and future recovery (45, 46). Here, we assessed the persistence of DNA markers in mesocosms in shaded conditions. This experimental design explores a scenario where persistence of microorganisms and DNA from sewage or recycled water is expected to be longer than persistence under full sun conditions. We contrasted our findings to other studies (Table 5) that explored decay of the same microbial variables tested in this and included freshwater mesocosms. We explored differences and similarities for conditions affecting the decay of DNA markers and highlight advantages of using culturable EcH8 to confirm the presence of untreated sewage in surface waters.

Factors Influencing the Decay of Microbes and DNA Markers in the Environment

Multiple abiotic and biotic factors can contribute to the decay of microbes and nucleic acids in environmental waters. The experimental design in this study included extrinsic microbial factors (i.e., predators and competitors), intrinsic factors (e.g., obligate and facultative anaerobes, viruses and bacteria), and measurements of water temperature and light intensity, all of which have been shown to be contributing factors for the decay of microbes and DNA markers in surface waters (reviewed in 24). In this study, we found a significant relationship between light intensity and decay of all nucleic acid markers (EC23S857, HF183 and CPQ_056) by multivariate analysis. However, we did not see any correlation between water temperature and decay of DNA markers.

Sunlight is an important contributor to the decay of microbes and DNA markers. Studies typically include measurements of light intensity and contrast full sun with complete shade (frequently termed a dark treatment) (Table 3.5). Many studies have shown a significant effect of sunlight on decay of DNA markers in a freshwater environment (25, 27, 29, 32). Sunlight was

the primary factor contributing to decay only in the first few days (days 2 – 5) while the presence of indigenous microbiota was the principal factor contributing to decay in the later stages (> 5 days) (25, 27, 29, 32). Reported light intensity in published studies ranges from 0 – 6,500 lum/ft.^{2}, while in our study we observed a smaller range of 0 – 3,400 lum/ft.^{2}, as experiments were conducted in natural shade. On the other hand, one study in the U.S. and another in Sweeden found no association between light exposure and decay of DNA markers (47, 48). Differences in the germicidal effect of sunlight in surface water can be partially explained by other studies that have shown the influence of multiple factors including but not limited to turbidity and water depth (49, 50), temperature and pH (51, 52), as well as concentrations of dissolved oxygen (53, 54) and humic acids (55, 56).

Multiple studies have found MST marker persistence to be greater at water temperatures below 20 °C (25, 47, 57-59). Water temperature was controlled in a previous study that demonstrated an effect of water temperature on decay of microbial variables (Table 5), in which the decay of HF183 and crAssphage CPQ_056 were greater at 25 °C compared to 15 °C in indoor mesocosms (25). Another study that tested decay with continuous-flow freshwater in a laboratory setting found that the time for a 99% reduction of DNA markers was significantly longer at 14 °C compared to 22 °C (57). The difference in mean water temperatures our study was only ~ 2 °C (~ 21 – 23 °C) across the three trials, which likely contributed to the lack of correlation with decay observed. Other studies in the U.S. have also found no association between water temperature and decay of microbes in outdoor and indoor mesocosms, with water temperatures ranging from 6 – 28 °C (33), and 15 – 28 °C (29).

Decay of DNA Markers from Recycled Water Versus Untreated Sewage

The microbiological material in properly treated recycled water is dominated by dead cells and free DNA, while that of untreated sewage contains a high proportion of viable cells. Because these sources of microorganisms and DNA have very different implications for human health risks, we compared their persistence, hypothesizing that the viable microorganisms in sewage would decay more slowly than the compromised cells and free DNA in recycled water. However, decay was slower in the recycled water treatment than in the sewage treatment. Our study also showed a greater time for one log reduction (T_{90}) for DNA markers HF183 and CPQ_056 in the recycled water treatment compared to the same markers in the sewage treatment. These trends were not expected and may be attributed to a greater influence of extrinsic factors (e.g., predation and competition) in the sewage treatment compared to recycled water. A greater population of microbes including protozoa in sewage mesocosms could explain some of these differences, as protozoan grazing can contribute up to 90% of mortality in microbial populations and extracellular DNA from lysed organisms can be consumed by metabolically active microbes (60, 61). To our knowledge there are no other studies on the environmental persistence of DNA markers from recycled water that can be used for comparison.

Decay of HF183 and CPQ_056 markers from the untreated sewage treatment were compared to trends reported in the literature. One study (25) conducted in an Australian lab with freshwater microcosms under artificial sunlight and a constant temperature of 25 °C found a lower range for HF183 first order decay rates and generally longer times to reach T_{90} compared to our study, but CPQ_056 decay and T_{90} values in this study were similar to our findings. However, Australian study was done in indoor microcosms with stable temperatures and used artificial lighting which emitted solar radiation that was approximately 6 times lower than natural outdoor conditions

(25). On the other hand, a study that deployed a mesocosm with untreated sewage in the upper Mississippi River (Iowa, U.S.) exposed to direct sunlight found greater $\log_{10}$ reductions (~ 4 $\log_{10}$ after 5 days) of HF183 compared to our study (~ 3 $\log_{10}$ after 5 days) but found similar decay for this marker in mesocosms under complete shade conditions (32). Another study in Indiana, U.S. also found greater decay rate for both HF183 and CPQ_056 in an outdoor unshaded mesocosm while the decay rates of both markers in complete shade were similar to ours (34). Intrinsic microbiological factors (i.e., physiology) logically have a greater contribution to differential decay in the sewage treatment, which is largely comprised of viable intact cells compared to recycled water that is mostly dead/compromised cells and free DNA.

Differential Decay Among Microbial Variables in Recycled Water or Sewage Treatments

Understanding how environmental persistence among DNA markers vary is important for identifying which markers can be used to detect recycled water or sewage over time. In this study we found that LRVs in the recycled water treatment were not significantly different among DNA markers, while LRVs of all DNA markers in the sewage treatment were significantly different from each other. To contrast, one study with untreated sewage found that CPQ_056 was more persistent compared to HF183 in a freshwater environment (25), which reflects results shown in our study. We found that HF183 decayed more rapidly than culturable *E. coli*, which was also reported in one other study (29). On the other hand, another study found no difference of decay between FIB DNA markers and HF183 but did find that culturable *E. coli* decayed faster than all DNA markers (32). The source of microbiological material (recycled water vs sewage) contributed to differential decay among DNA markers.

Persistence of Culturable EcH8 in the Environment

Recreational water quality methods such as MST are analyzing all the DNA in a water sample to identify markers of sewage but fail to address implications of qPCR methods that also detect DNA from dead cells and extracellular DNA from treated wastewater and the environment. Therefore, a culturable marker of untreated sewage is one possible path to circumvent limitations of methods that use qPCR alone. We composited *E. coli* to test for the H8 gene as this increased the method sensitivity for experiments but only provided persistence data as frequency of detection. Persistence of culturable EcH8 in contaminated surface waters is limited by the physiology of *E. coli* as well as their ability to survive and remain culturable while exposed to environmental pressures over time. Organic carbon and phosphorus are two examples of limiting nutrients, and when present at higher concentrations both can enhance survival of *E. coli* in surface waters (62-65). Although elevated levels of nutrients in surface waters can also alleviate pressures from extrinsic factors (i.e., predation and competition) as shown in previous studies (65, 66), many aquatic environments are considered oligotrophic compared to gastrointestinal tracts where enteric bacteria originate. Our study demonstrated that culturable EcH8 can persist in river water and remain detectable as long as other DNA markers in a shaded freshwater environment, which supports what we originally hypothesized. Culturable EcH8 has strong potential to confirm recent pollution events of sewage in shaded freshwater environments.

Detecting culturable EcH8 is a strong indication that surface waters were recently contaminated with untreated sewage; on the other hand, the absence of culturable EcH8 does not rule out the presence of untreated sewage, as *E. coli* may have died or entered a viable but non-culturable state when exposed to environmental pressures. Our findings show that the average LRV of culturable *E. coli* was 0.38 after 5 days in shaded river water spiked with untreated sewage. A

similar trend was found for culturable *E. coli* in two previous studies with untreated sewage in freshwater samples in complete shade (26, 32). However, a greater decay (> 2 $\log_{10}$ reduction) after 5 days for *E. coli* was demonstrated in other studies with untreated sewage spiked freshwater exposed to direct sunlight (26, 32, 34). Furthermore, decay of *E. coli* from sewage was substantially greater (> 3 $\log_{10}$ reduction) after 5 days in marine water compared to freshwater in both shaded and unshaded conditions (26, 27). These findings provide examples of different scenarios in which environmental persistence of *E. coli* can vary and present a possible challenge when relying on culturing to test for the H8 genetic marker in the environment. Despite this challenge, advantages of testing for culturable EcH8 include the ability to confirm a recent contamination event of untreated sewage and improve health risk assessments by targeting a viable indicator in surface waters that is only from untreated sewage and not recycled water.

Conclusions

- Multivariate analyses showed that the source of microbial cells and DNA (recycled water or untreated sewage) was a significant factor in decay of DNA markers, which was greater in river water spiked with untreated sewage compared to recycled water.
- Redundancy analysis demonstrated a relationship between light intensity and the $\log_{10}$ reduction of DNA markers in a simulated freshwater environment.
- The decay rate among DNA markers were similar in river water spiked with recycled water; however, decay rates were very different among markers from untreated sewage.
- Culturable EcH8 was able to persist and remain detectable in river water spiked with untreated sewage and was comparable to the detection of DNA markers.

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Table 3.1. *P* values for two-factor ANOVA on the effect of factors (trials and markers) on $\log_{10}$ reductions in recycled water or untreated sewage treatments (n=3). *P*-values < 0.05 are bolded.

Factors	Recycled Water	Untreated Sewage
Trials	**0.0030**	< **0.0001**
DNA Markers	0.9044	< **0.0001**
Trials : DNA Markers	0.4951	< **0.0001**

Table 3.2. *P* values for ANOVA on linear mixed effect model to test the effect of source (recycled water or untreated sewage) on $\log_{10}$ reductions in river water spiked with recycled water or untreated sewage (n=3). *P*-values < 0.05 are bolded.

Factors	EC23S857	HF183	CPQ_056
Source (Recycled Water vs Untreated Sewage)	0.1310	0.1369	0.1095

Table 3.3. Decay rates (*k*) and fit of log linear regression model for microbial variables in river water spiked with recycled water or sewage.

Recycled Water Treatment	Trial 1				Trial 2				Trial 3			
	k	T_{90}	R^2 Adj.	RMSE[a]	*k*	T_{90}	R^2 Adj.	RMSE	*k*	T_{90}	R^2 Adj.	RMSE
EC23S857	- 0.20	11.5	0.59	0.13	- 0.28	8.1	0.19	0.31	- 0.60	4.2	0.65	0.33
HF183	- 0.19	12.2	0.59	0.12	- 0.31	7.4	0.63	0.15	- 0.64	4.1	0.61	0.38
CPQ_056	- 0.20	11.3	0.32	0.18	- 0.21	11.0	0.13	0.51	- 0.14	16.9	0.14	0.41
Sewage Treatment												
EC23S857	+ 0.19	NA	NA	0.31	- 0.21	11.1	0.35	0.43	- 0.18	12.9	0.45	0.32
HF183	- 0.35	6.7	0.91	0.09	- 1.3	1.8	0.91	0.33	- 1.37	1.7	0.89	0.42
CPQ_056	- 0.27	8.6	0.69	0.14	- 0.56	4.5	0.70	0.24	- 0.46	4.9	0.72	0.23

[a] RMSE = Root Mean Sum of Squared Errors.

Table 3.4. Frequency of detection for culturable EcH8 (n=5) for each day in river water spiked with recycled water or untreated sewage across three experimental trials.

Days	Recycled Water			Untreated Sewage		
	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
Day 0	< LOD[a]	< LOD	< LOD	100 %	100 %	100 %
Day 1	< LOD	< LOD	< LOD	100 %	80 %	100 %
Day 2	< LOD	< LOD	< LOD	80 %	80 %	100 %
Day 3	< LOD	< LOD	< LOD	80 %	60 %	80 %
Day 4	< LOD	< LOD	< LOD	40 %	80 %	20 %
Day 5	< LOD	< LOD	< LOD	60 %	60 %	40 %

[a] < LOD = below limit of detection.

Table 3.5. Comparison of experimental design in previously-published studies on environmental persistence of fecal microorganisms and their DNA sourced from untreated sewage. All studies included natural predators and competitors.

Variable(s)	Environment	Sunlight	Shade	Source of Microbes and DNA	Decay Metric	Factors Tested	Significant Factors	Citation
E. coli, HF183, CPQ_056	Laboratory	Artificial UV	No shade	Predators & competitors	T_{90} & k	Temperature & natural biota	Temperature	(25)
E. coli	Laboratory & outdoor mesocosm saline water	Natural	No shade & dark treatment	Predators & competitors	T_{90}	Temperature	None	(33)
E. coli, HF183	Laboratory	Artificial UV	No shade & dark treatment	Predators & competitors	T_{99}	Temperature, predation, & artificial sunlight	Predation	(29)
E. coli, HF183	Outdoor mesocosm saline water	Natural	No shade & complete shade	Predators & competitors	Log_{10} reduction	Microbiota & sunlight exposure	Microbiota & sunlight	(27)
E. coli, HF183	Outdoor mesocosm fresh water	Natural	No shade & complete shade	Predators & competitors	Log_{10} reduction	Microbiota & sunlight exposure	Microbiota & sunlight	(32)
E. coli, HF183, CPQ_056	Outdoor mesocosm fresh water	Natural	No shade & complete shade	Predators & competitors	k	Sunlight & microbiota	Sunlight & microbiota	(34)
E. coli, HF183	Outdoor mesocosm fresh water	Natural	No shade & complete shade	Predators & competitors	Log_{10} reduction	Sunlight & fecal source	Fecal source	(48)
Enterococci, *E. coli*, HF183	Outdoor mesocosm fresh water	Natural	No shade & complete shade	Predators & competitors	T_{90} & k	Sunlight, microbiota, & season	Season	(47)

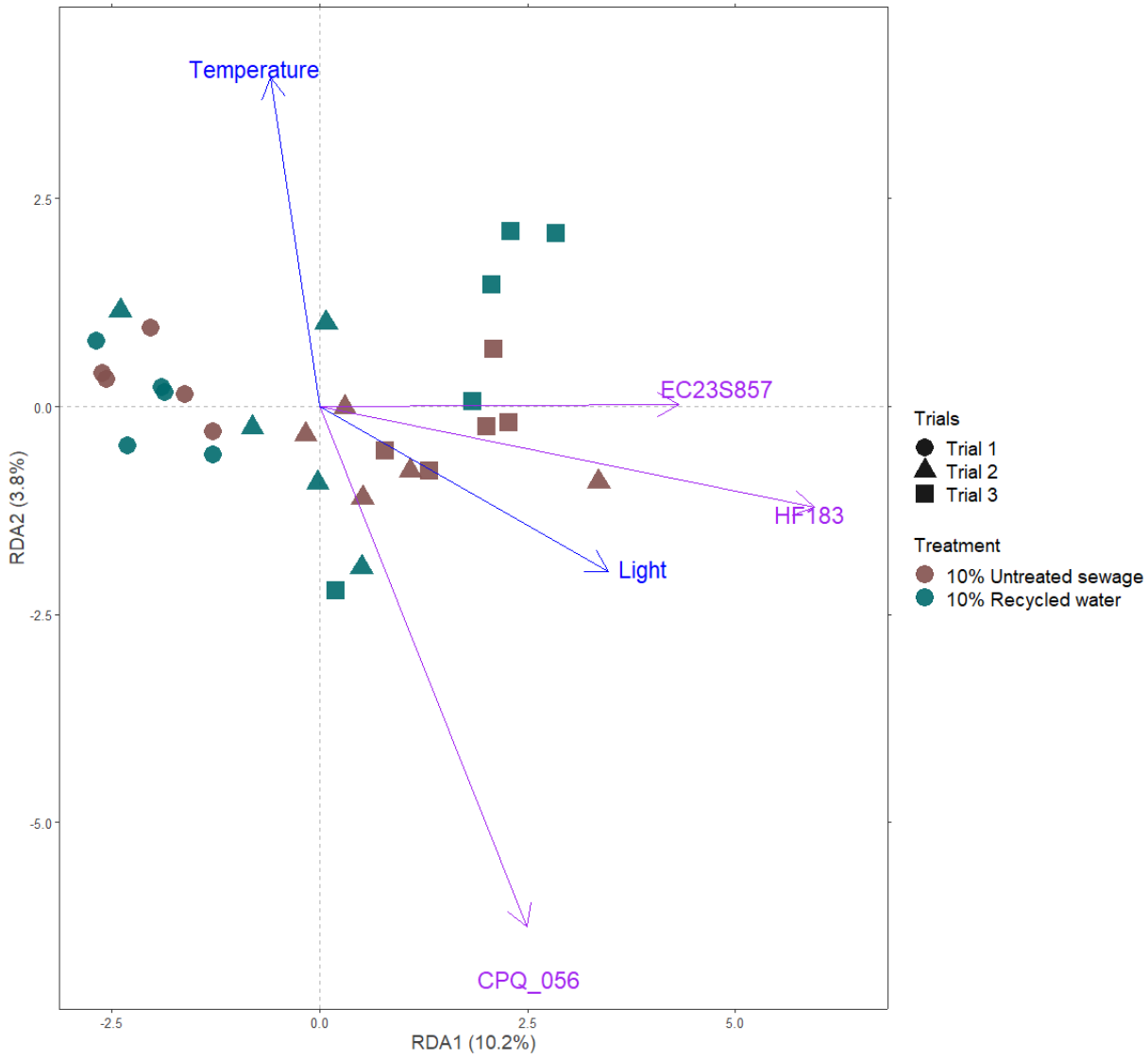

Figure 3.1. Relationships among environmental variables and decay (log_{10} reduction) of DNA markers measured by qPCR and analyzed by redundancy analysis. Ordination plot of recycled water (teal) or untreated sewage (brown) spiked river water data showing microbial variables (pink arrows) and environmental variables (blue arrows). Data points for trials are shown as circles (Trial 1), triangles (Trial 2), and squares (Trial 3). The first axis (horizontal) explains 10.2 % of the variability, while RDA axis II (vertical) explain 3.8 % of the variability observed.

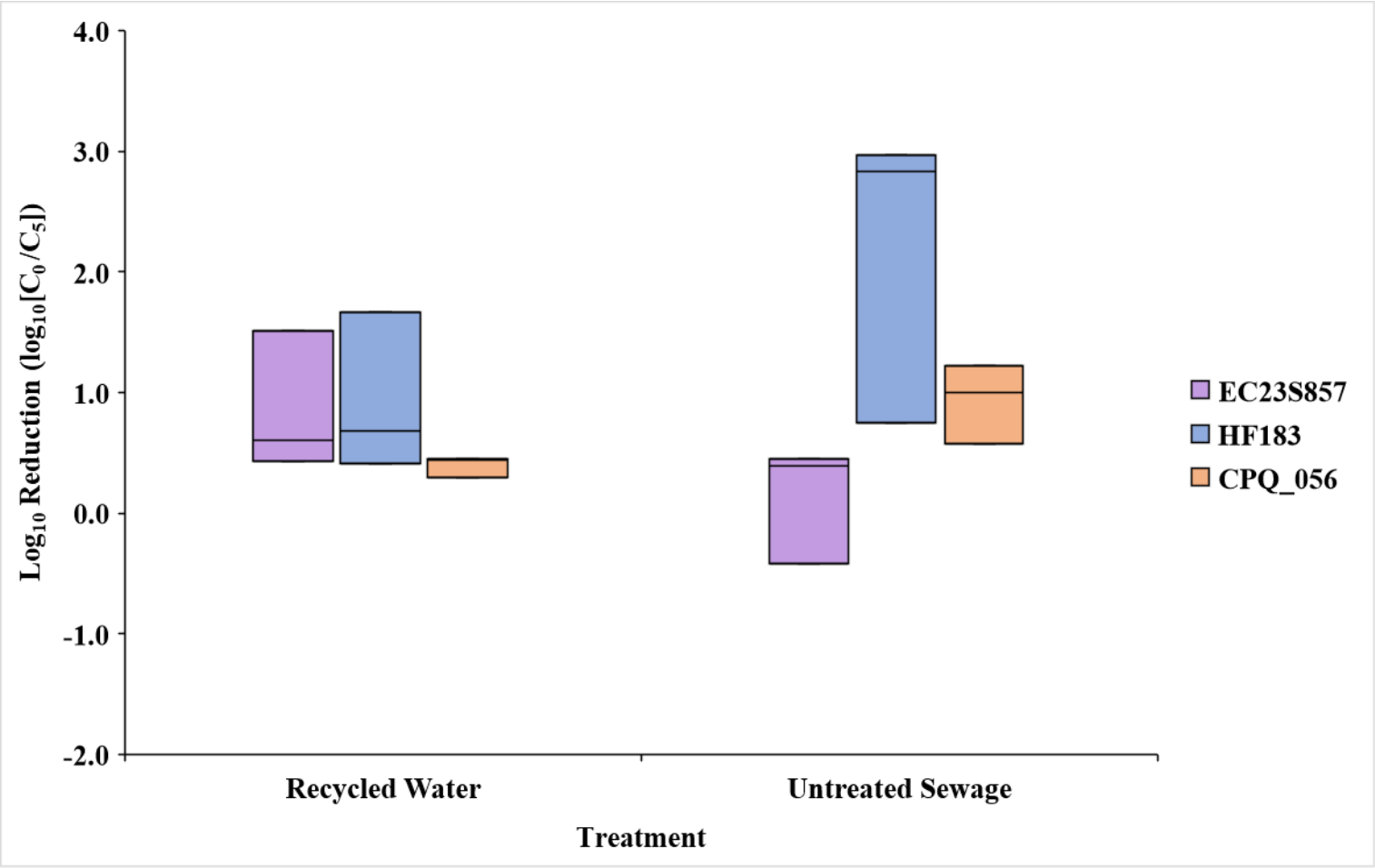

Figure 3.2. Decay (log_{10} reduction) of marker genes over 5 days measured by qPCR in river water spiked with recycled water or untreated sewage (n=3). Black bars represent median concentrations; boxes show the min and max values of observations.

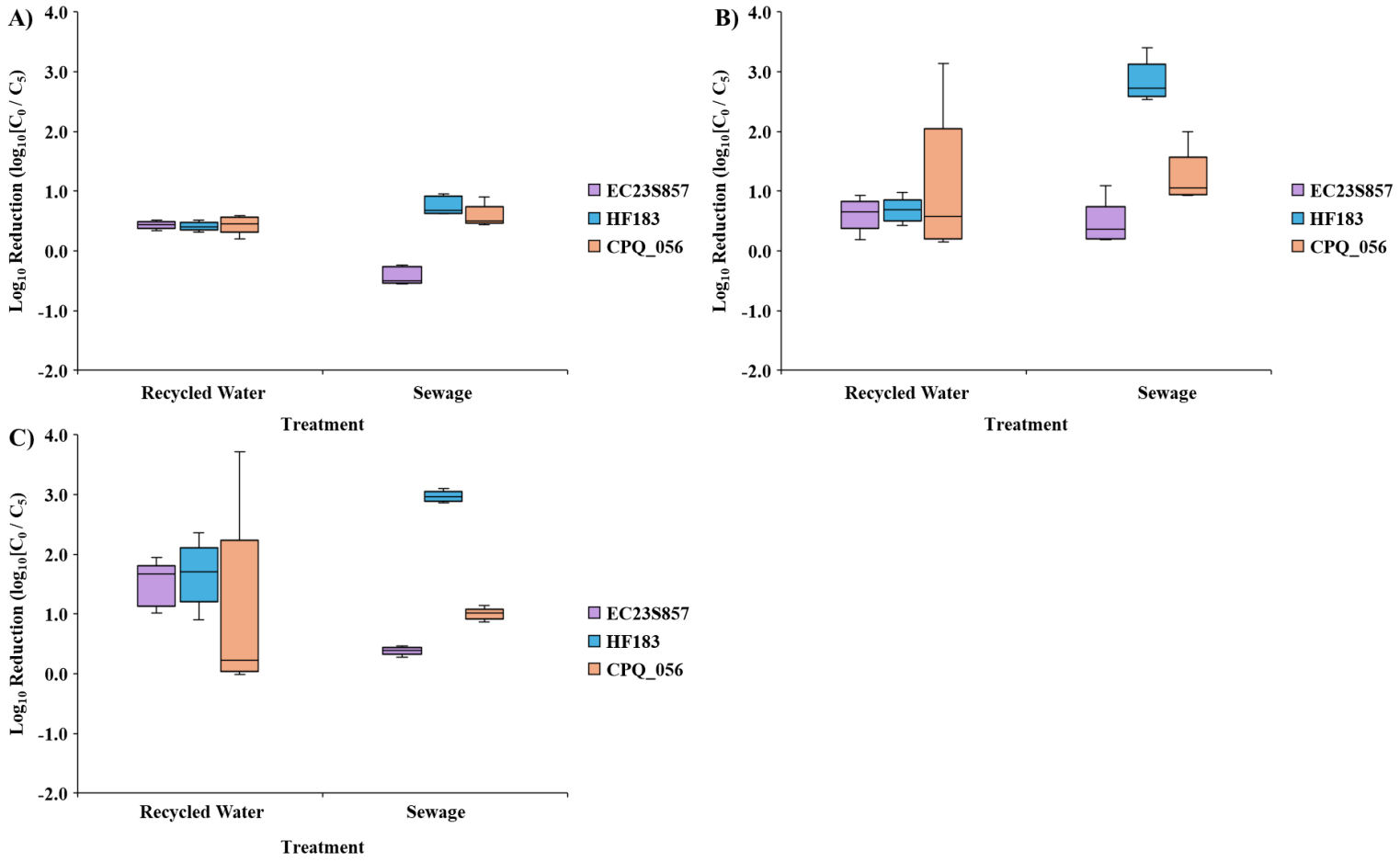

Figure 3.3. Differential decay of DNA markers between recycled water or untreated sewage treatments with $\log_{10}$ reductions of the general *E. coli* marker EC23S857, and sewage-associated markers HF183 and CPQ_056 measured by qPCR from 5-day experiments conducted on three separate trials A (March), B (April), and C (May) (n=5). The interquartile range (25^{th} and 75^{th} percentile) includes the LRV medians (black horizontal bar) and boxplot whiskers represent the 10^{th} and 90^{th} percentile values.

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CHAPTER FOUR: DOES EXTRACELLULAR DNA FROM TREATED WASTEWATER HAVE POTENTIAL TO INFLUENCE MICROBIAL ANALYSES OF RECREATIONAL WATER QUALITY?

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Abstract

Standard quantitative PCR methods for assessment of recreational water quality measure DNA markers to indicate increased probability of viable pathogens in surface waters. However, studies that utilize these methods ignore the possibility that extracellular DNA (exDNA), which has no known association with human health risk, could interfere with data interpretation. We utilized intact cells and exDNA from pure cultures of *Escherichia coli* (DNA marker, EC23S857) and *Enterococcus faecalis* (DNA marker, Entero1A), and exDNA from a pure culture of a haloalkaliphilic archaeon *Natronomonas pharaonis* (DNA marker, NP*gyr*A) that was used for spiking river water and recycled water. Following standard recreational water quality methods, we filtered solutions of intact cells, exDNA, river water spiked with exDNA (river water treatment), recycled water spiked with exDNA (recycled water treatment), and recycled water without treatment (HF183 experiment) . Samples were analyzed by qPCR to determine the

percentage of gene copies or total gene copies (for HF183) that were captured on polycarbonate membranes and eliminated by DNase I treatment. Filtered solutions of intact cells resulted in similar percentages among bacterial markers (80 – 86%) of DNA captured on membrane filters. DNase I treatment did not negatively affect gene copies captured from intact cells; however, treatment did result in growth for both *E. coli* and *Ent. faecalis* on membrane filters. For filtered solutions of exDNA derived from lysed cells of pure cultures, the percentage of EC23S857 (*E. coli*) gene copies captured on membrane filters was significantly greater than that of Entero1A (*Ent. faecalis*), although mean percent capture was low for EC23S857 (1.4%) and Entero1A (0.5%). DNase I treatment effectively eliminated the qPCR signal of exDNA from pure cultures of *E. coli* and *Ent. faecalis*. Analysis of river water and recycled water treatments showed that approximately 1% of NP*gyr*A gene copies were captured on membrane filters. Exposure to DNase I significantly reduced gene copies captured but did not eliminate the qPCR signal in each sample, as more than 10^2 gene copies captured on filters were quantified by qPCR for each treatment. Testing for HF183 in recycled water revealed that the HF183 signal on membranes captured from recycled water was reduced from ~ 4 $\log_{10}$ down to ~ 3 $\log_{10}$ gene copies following DNase I treatment. The percentage of exDNA in environmental water captured by recreational water quality filtration methods was low (< 2%) but may still provide a signal that can be interpreted as a false indication of fecal contamination, particularly if exDNA concentrations are greater than 10^4 GC/100 mL. DNase I treatment of membranes is a potentially useful strategy to reduce the amount of extracellular DNA that is captured and analyzed by recreational water quality methods.

Introduction

Extracellular DNA (exDNA) in recreational waters may lead to an overestimation in public health risk for studies that utilize recreational water quality techniques containing a filtration step that is designed to concentrate bacteria. Standard methods to assess recreational water quality in the United States generally measure fecal indicator bacteria (FIB) such as *Escherichia coli* (1) and enterococci (2) as surrogates that indicate heightened risk of the presence of pathogens in surface waters. Assessing viable pathogens in surface waters is complex, as pathogen diversity in feces and sewage is high, while pathogen concentrations in the environment are generally very low (3-5), which is why FIB are commonly used as a proxy for pathogens. Recreational water quality criteria also include a qPCR method developed by the US Environmental Protection Agency (EPA), Method 1611 targeting the DNA marker Entero1A, which quantifies DNA from *Enterococcus* spp. in recreational waters (6). Microbial source tracking (MST) is another example of a recreational water quality method that relies on measuring DNA by qPCR in surface waters. If sewage is the suspected source of fecal contamination, HF183 is generally used (4, 7).

Recreational water quality methods typically concentrate bacteria and viruses by membrane filtration (8-13), but many studies do not address the implications of captured exDNA for human health risk estimates. DNA extracted from microbes captured by membrane filtration is frequently analyzed to inform quantitative microbial risk assessments (14-17). However, qPCR alone lacks the ability to determine if cells are viable, which can be an issue, since risk is associated with the presence of viable and infectious pathogens. The extent to which exDNA in surface water is captured by recreational water quality techniques is a major knowledge gap and could misinform regulatory agencies and human health risk estimates since qPCR techniques

simply detect nucleic acid. Studies found in the literature show that exDNA can persist in environmental conditions and accumulate over time in freshwater and marine environments (18-19). Sources of exDNA that may impact estimates of human health risk in recreational waters can include lysed microorganisms from treated wastewater effluent, recycled water, stormwater runoff, and the environment.

Treated wastewater effluent and recycled water containing exDNA has potential to provide a false indication of fecal contamination (e.g., sewage pollution) in surface waters qPCR is the sole method used to detect fecal contamination (20). Disinfection of sewage with chlorine or ultraviolet radiation is generally an effective means of inactivating pathogens (21-23), but multiple studies have demonstrated the persistence of exDNA in wastewater effluent and recycled water (20, 24-28), and that surface water discharge can increase concentrations to > 1,000 GC/100 mL of HF183 in a freshwater stream (20). Production of treated effluent and recycled water in wastewater treatment facilities exceeding facility capacity, which is typically millions of gallons per day, is often directly discharged into surface waters (29). The proportion of exDNA in recycled water that is captured by recreational water quality techniques is poorly understood, and should be explored to determine whether exDNA is a major hindrance to the accuracy of DNA-based estimates of human health risk from exposure to recreational water.

A major concern for recreational water quality studies is with how much exDNA originally from bacteria and viruses may be captured on membranes, and should not be confused with environmental DNA, which other studies collect and analyze by different methods (30-33). Recreation water quality methods generally require filtration of water samples with membranes engineered to contain pores of precisely 0.45 μm in diameter, followed by DNA extraction with a commercial kit (e.g., Qiagen DNeasy PowerWater) that includes a mechanical/chemical lysis

step (8-13, 34). One study demonstrated that roughly 9% of exDNA was captured from stormwater through a process similar to recreational water quality techniques described above (35). However, this study measured the capture of plasmid DNA (*Clostridium parvum* 18S rRNA gene fragment) contained in stormwater on a 0.2 μm polycarbonate membrane filter and did not examine the effect of a larger pore size filter (i.e., 0.45 μm) (35). A greater understanding of how much exDNA from recycled water and environmental water is captured by recreational water quality methods will clarify possible limitations for data interpretation, and the need for a modified approach.

Methods for differentiating exDNA and compromised cells from intact cells in qPCR analyses have been explored, and while some partially mitigate exDNA interference, all have limitations. Ethidium monoazide (EMA) or propidium monoazide (PMA) treatment prior to qPCR (36), quantitative reverse transcriptase PCR (qRT-PCR) (37-38), and inversely-coupled immunomagnetic separation and adenosine triphosphate (Inv-IMS/ATP) quantification (39) are all examples of methods used in previous studies to differentiate the qPCR signal in viable targets from that of dead cells and exDNA in water samples. Some studies show that photoreactive DNA-binding dyes can lead to an underestimation (EMA) or overestimation (PMA) of viability, since EMA can penetrate intact cell membranes and PMA can fail to bind to all exDNA, or to DNA in dead bacteria (36, 40-43). QRT-PCR methods based on messenger RNA quantification faces limitations due to rapid degradation in environmental samples (which can be caused by nucleases and abiotic factors), and transcript abundance can vary among genes and phases of cell growth (37-38, 44). The measurement of ATP in *Bacteroides thetaiotaomicron* by Inv-IMS/ATP methods also have major limitations for environmental matrices, as sensitivity can be impacted by turbidity in water samples (39). Therefore, an alternative approach to

eliminate all exDNA without impacting intact viable cells in environmental samples will be necessary to effectively inhibit the qPCR signal from recycled water and other eDNA sources.

One promising approach to eliminate exDNA is the use of the nonspecific endonuclease deoxyribonuclease I (DNase I) to digest exDNA, as it cleaves single- and double-stranded DNA. Although environmental factors can in some cases inhibit the enzyme activity of DNase I (45-46), recent studies have explored treating membrane filters directly to mitigate interference and demonstrated a relatively effective process to eliminate the majority of exDNA in a sample (47-48). In theory, the DNA in viable cells should not be impacted by DNase I as they have protection from intact cell walls; however, DNA in cells with compromised membranes and any exDNA present in a sample will be digested by the enzyme. The literature contains scant information on how DNase I treatment on membrane filters may impact the qPCR signal from intact cells (i.e., *E. coli* or enterococci) and whether this method is reliable for removing the qPCR signal from exDNA in recreational water quality studies. It is important to understand how DNase I treatment may impact both gram-positive and gram-negative bacteria, as any negative effects will lead to an underestimation of viable cells. This approach may offer a path to determine how much exDNA is captured from recycled water and surface water by recreational water quality techniques and if this method can be applied to eliminate any exDNA persisting on membranes.

The main objective of this study was to examine the proportion of exDNA or intact cells captured on filters by recreational water quality methods, while exploring the effectiveness of DNase I treatment to eliminate exDNA, but not DNA from intact cells (Figure 4.1). Capture of exDNA and intact cells from pure cultures of gram-positive (*Enterococcus faecalis*, Entero1A) and gram-negative (*Escherichia coli*, EC23S857) bacteria, which are FIB commonly targeted in

the recreational water quality field, was measured by qPCR. Furthermore, exDNA of lysed cells from a pure culture of the extreme halophile *Natronomonas pharaonis* (NP*gyr*A), which is absent from river water and recycled water, was used to spike these water types in experiments also designed to assess capture efficiency by qPCR. We tested two hypotheses: (i) exDNA derived from pure cultures can be captured and quantified by standard recreational water quality techniques, and (ii) DNase I treatment eliminates the persistent qPCR signal from exDNA in recycled water or surface water, but does not harm intact cells.

Methods

Growth of Pure Cultures and Preparation of exDNA

Ent. faecalis (ATCC 19433™) was cultivated on BD Difco™ membrane-*Enterococcus* indoxyl-β-D-glucoside (mEI) agar according to USEPA Method 1600 (2), while *E. coli* (ATCC 11775™) was cultivated on BD Difco™ modified membrane- thermotolerant *Escherichia coli* (mTEC) agar following USEPA Method 1603 (1). Colonies of *E. coli or Ent. faecalis* were collected with a sterile loop and inoculated into 50 mL of brain heart infusion (BHI) broth in a sterile 250 mL Erlenmeyer flask. Broth cultures were incubated at 41 °C in an Innova 4000 shaker incubator (New Brunswick Scientific Co.; Brunswick, NJ) that maintained shaking at 180 rev/min for ~18 hours. Overnight cultures of *E. coli and Ent. faecalis* were used to prepare intact cells (i.e., in log-phase) or exDNA for membrane filtration experiments. *N. pharaonis* (DSM 2160 ™) was cultivated in NP media following a protocol made by Southern California Coastal Water Research Project (34). NP media broth was comprised of NaCl (200 g/L), KH_2PO_4 (1 g/L), NH_4Cl (1 g/L), $MgSO_4$ x $7H_2O$ (0.24 g/L), $CaSO_4$ x $2H_2O$ (0.17 g/L), yeast extract (5 g/L), glucose (1 g/L), casamino acids (5 g/L), Na_2CO_3 (5 g/L), SL-10 trace elements (100 µL/L). Overnight culture of *N. pharaonis* was prepared in a sterile 125 mL Erlenmeyer flask with 50 mL

of NP media and incubated at 37 ℃ in an Innova 4000 shaker incubator (New Brunswick Scientific Co.; Brunswick, NJ) that maintained shaking at 180 rev/min.

For intact cells in log-phase growth, 100 µL of overnight cultures of *E. coli* or *Ent. faecalis* were added to 50 mL of BHI broth in a sterile 250 mL Erlenmeyer flask and incubated at 41 ℃ in an Innova 4000 shaker incubator (New Brunswick Scientific Co.; Brunswick, NJ) that maintained shaking at 180 rev/min for 100 min. Six replicates of a 10^{-2} dilution was prepared for cultures with cells in log-phase of growth and 50 µL aliquots were spiked into 950 µL of phosphate buffered saline (PBS, pH 7.1) and immediately used in experiments.

Preparation of exDNA was achieved by preparing six replicates of 60 µL aliquots of a 10^{-3} dilution of overnight cultures (*E. coli*, *Ent. faecalis*, and *N. pharaonis*) and boiling in sterile 100 µL microcentrifuge tubes for 10 min at 100 °C in a Bio-Rad thermal cycler. Boiled samples were then centrifuged at 13,000 g for 1 min to pellet cellular debris; exDNA was recovered in the supernatant and 50 µL was spiked into 950 µL of PBS (pH 7.1) for *E. coli* or *Ent. faecalis*, and for *N. pharaonis* exDNA, 50 µL was spiked into 950 µL of river water or recycled water. All spiked solutions were immediately used in experiments.

Recycled Water and River Water

The proportion of exDNA captured during filtration of recycled water and river water was analyzed to determine whether exDNA in those matrices could impact recreational water quality analyses and if DNase efficacy would be affected by the presence of environmental factors. River water and recycled water were spiked with an exogenous DNA target (NP*gyr*A) from the lysed cells of *N. pharaonis*, which was utilized as it is not present in river water or recycled water and would therefore not represent a source of intact cells in the experiment. This DNA marker was used to measure the percentage of gene copies from lysed cells (exDNA) that could

be captured on membranes following filtration of river water or recycled water. Recycled water was collected from a conventional wastewater treatment facility in St. Petersburg Florida, and river water was collected from the Hillsborough River (28.088007, -82.348996) in Tampa, Florida. Approximately126,880 residents are serviced by the wastewater treatment facility and ~16 million gallons of recycled water is generated daily. Recycled water was produced by an activated sludge process including primary and secondary treatment of sewage, and disinfection of effluent was achieved with sodium hypochlorite. Recycled water (500 mL) and river water (500 mL) were collected in sterile 1 L polypropylene containers which were transported at 4 °C on wet ice to the laboratory on the same day of experiments. Holding time of samples was < 2 hours before they were processed by membrane filtration and DNA extraction as described below.

Membrane Filtration and DNA Extraction

Prepared solutions (1 mL) included six replicates of intact cells or eDNA for each treatment and were filtered and concentrated on polycarbonate filters (47 mm diameter 0.45 µm pore size; MilliporeSigma™ HTTP04700). One set (n=6) of filter membranes were saved for DNase I treatment, which is described in the section below, while the other set of membranes were processed in a DNeasy® PowerWater® (Qiagen) DNA extraction kit according to the manufacturer instructions, and 100 µL of purified DNA was eluted. The filtrate of samples from each experiment was collected in a sterile 125 mL vacuum Erlenmeyer filter flask and transferred into sterile 1.5 mL Eppendorf tubes that were saved on wet ice at 4 °C until qPCR analysis, holding time was < 30 minutes.

DNase I Treatment of Filter Membranes

The DNase treatment procedure was modified from methods described in previous studies (47-48). Following filtration of each sample, an individual membrane was placed into a sterile, 47 mm Petri dish, and 2 mL of DNase solution was added to submerge the membrane. Solutions were prepared with DNase I (Thermo Scientific™) at a final concentration of 0.1 U/µL, and a 10× reaction buffer (final concentration: 10 mM Tris–HCl, 2.5 mM $MgCl_2$, and 0.1 mM $CaCl_2$). Filters in Petri dishes with lids on were incubated at 37 ℃ for 30 minutes in an Innova 4000 shaker incubator (New Brunswick Scientific Co.; Brunswick, NJ) that maintained shaking at 120 rev/min. Following incubation, the membrane and all the solution in the Petri dish was transferred into bead beating tubes from a DNeasy® PowerWater® (Qiagen) extraction kit. DNA was extracted according to manufacturer's instructions.

Quantitative PCR Analyses

Quantitative PCR assays included the general *Enterococcus* 23S ribosomal RNA gene Entero1A (6), the *E. coli* multi-copy 23S rRNA gene EC23S857 (49), the *gyr*A gene in *N. pharaonis* NP*gyr*A (34), and sewage-associated HF183 (7). Sequences and concentrations of primers and probes as well as qPCR run conditions are reported in Table 4.1. QPCR amplification was conducted in 25 µL reactions in triplicate using 12.5 µL TaqMan Environmental Master Mix 2.0 (Applied Biosystems) and 5 µL of template DNA per reaction in a Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories). Standard curves were constructed from gene fragments in gBlocks containing the target sequences. Reference DNA material (internal amplification control) was included in samples for the HF183 assay to test for inhibition in river water and recycled water samples according to guidelines in USEPA method 1696 (7). Inhibition of qPCR amplification was not detected in this study. The dynamic range of standard curves for

EC23S857, Entero1A, and NP*gyr*A was from 5 to 10^6 gene copies per reaction and performance metrics included efficiencies between 90% and 110%, and > 0.98 R^2 values. For DNA captured on membranes, the limit of detection (LOD) and the limit of quantification (LOQ) was 100 GC/mL for each DNA marker. For DNA tested in the filtrate, the LOD and LOQ was 1,000 GC/mL. Negative controls for each instrument run included 3 extraction blanks, and 3 non-template controls (nuclease free water used in qPCR) which were all negative for each qPCR target in this study. The *N. pharaonis* DNA marker (NP*gyr*A) was below the LOD in Hillsborough River water and recycled water prior to the spiking experiment.

Data Analysis

Data were analyzed using Microsoft Excel version 2404 and R version 4.4.0 (50). All figures were constructed in Microsoft Excel and statistical analyses were executed in R with the package stats (version 4.1.3). Percentage of gene copies (GC) captured (Pc) was calculated by:

$$Pc = \left(\frac{GC_b}{GC_a}\right) \times 100$$

Where 'GC_a' is the average gene copies measured prior to filtration, and 'GC_b' is the gene copies measured following filtration in the filtrate or membrane samples. To test differences between percentages of gene copies captured on membranes, an ANOVA was performed with Pc as the dependent variable and microbe (i.e., *E. coli* and *Ent. faecalis*) or treatment (with or without DNase I) as the independent variable. For analysis of HF183 data, the Pc for HF183 could not be calculated for recycled water as it was not known how many gene copies were present prior to filtration; therefore, the effect of treatment was tested on total HF183 gene copies captured on membranes with and without DNase I treatment by ANOVA. HF183 was not detected in river water samples.

Results

Effect of DNase I Treatment on Intact Cells from Pure Cultures Captured on Membranes

The percentage of gene copies in the filtrate and captured on membranes with and without DNase I treatment following filtration was analyzed using pure cultures of *E. coli* and *Ent. faecalis* in log-phase of growth and measuring DNA markers by qPCR for EC23S857 and Entero1A, respectively. Less than 2% of the qPCR signal was present in all filtrate samples, while an average of 80 – 86% was captured on the membrane which increased to 108 – 170% following DNase I treatment (Figure 4.2, Table 4.2). There was no significant difference between the percentage of gene copies for EC23S857 and Entero1A captured on membranes (Table 4.3). However, the percentage of gene copies captured were significantly greater on membranes with DNase I treatment compared to membranes without treatment for each DNA marker (Table 4.3).

Capture of exDNA from E. coli and Ent. faecalis on Membranes, and Elimination by DNase I Treatment

Cells from pure cultures of *E. coli* and *Ent. faecalis* were lysed for exDNA which was filtered to test the percentage of gene copies captured in the filtrate and on membranes with and without DNase I treatment by measuring DNA markers for EC23S857 and Entero1A by qPCR. Most of the qPCR signal from exDNA passed through membranes and was present in the filtrate (> 50%), while an average of ~ 1% was captured on the membrane and was below our LOD for membranes with DNase I treatment (Figure 4.3, Table 4.2). The percentage of EC23S857 gene copies captured on membranes was significantly greater compared to Entero1A when filtering exDNA (Table 4.3). Furthermore, the percentage of gene copies captured for both DNA markers were significantly greater on membranes without treatment compared to membranes with DNase I (Table 4.3).

Capture and Elimination of exDNA in River Water and Recycled Water on Membranes

Cells from a pure culture of the haloalkaliphilic archaeon *N. pharaonis* was lysed for exDNA that was spiked into river water and recycled water, which was filtered to test the percentage of gene copies captured in the filtrate and on membranes with and without DNase I treatment by measuring the DNA marker NP*gyr*A by qPCR. We also analyzed total gene copies of the sewage-associated DNA marker HF183 that was captured in the filtrate and on membranes with and without DNase I treatment following filtration of river water and recycled water. For both river water and recycled water, > 35% of the qPCR signal measured with NP*gyr*A was present in the filtrate, while < 2% was captured on membranes and an average of 0.03% persisted on membranes following DNase I treatment (Figure 4.4, Table 4.4). DNase I treatment significantly reduced the percentage of NP*gyr*A gene copies captured when comparing membranes with and without treatment in river water and recycled water. Furthermore, the total gene copies of sewage-associated HF183 captured on membranes was significantly reduced when exposed to DNase I treatment (Table 4.4).

Discussion

The effect of exDNA on human health risk estimates and false identification of untreated sewage is a factor often omitted in recreational water quality studies. DNA from bacteria and viruses are often analyzed in surface waters (9-13, 34), where concentration techniques typically involve membrane filtration which can capture not only DNA from intact microorganisms but may also capture all DNA in a sample including exDNA (35, 47). Detecting DNA markers from captured exDNA in recreational water samples can complicate data interpretation especially when relying on methods such as EPA method 1609.1 (6) or EPA method 1696 (7) to indicate the presence of viable pathogens originating from feces or untreated sewage. Understanding the extent to which

exDNA is captured by recreational water quality techniques will help determine the need for methods to eliminate or circumvent exDNA interference when targeting DNA from intact cells. In this study we provide data on the amount of exDNA captured by standard recreational water quality methods and demonstrate the usefulness of DNase I to attempt elimination of exDNA in river water and recycled water.

Our findings supported our first hypothesis, where roughly 1% of exDNA in solution was captured on polycarbonate membrane filters (0.45 µm pore size) when river water or recycled water was filtered following recreational water quality techniques. Another study found that 1-2% of exDNA was captured by 0.45 µm pore size polycarbonate membranes, while the percent captured was greater for membranes with a smaller pore size (i.e., 0.1 and 0.2 µm) and on membraned comprised of mixed cellulose ester (35). This other study utilized plasmid DNA (*Clostridium parvum* 18S rRNA gene fragment) which was suspended in DI water prior to membrane filtration; therefore, this study did not demonstrate the percentage of exDNA that can be captured from environmental water following membrane filtration (35). The other study and our study used different DNA extraction kits (DNeasy PowerWater vs PowerSoil) but both extraction methods are commonly used in recreational water quality studies and implement a bead-beating lysis step (51-54). Although the percentage of exDNA captured by these methods is low (< 2%), it can be enough to confound results in studies that aim to detect untreated sewage or fecal contamination in surface waters (20).

The application of DNase I to eliminate exDNA captured on membrane filters was to our knowledge only demonstrated in two other studies involving bacteria (47-48). Our study showed that DNase I treatment on membrane filters significantly reduced the total gene copies by ~ 2 $\log_{10}$. A study on drinking water reflected our results by demonstrating a 2 – 4-fold reduction of

exDNA on membranes following DNase I treatment and quantification of GC by qPCR (47). Another study focused on drinking water used PCR to show showed that DNase I completely digested 1 ng/µL exDNA derived from *Ent. faecalis* (48). This study found that enzyme efficiency was dependent on the concentration of DNase I and membrane composition, with optimal performance using 0.25 U/µL of DNase I on polycarbonate filters (48), while our study used a DNase concentration of 0.1 U/ µL. This filter type was also used in our study and is typically used in standard recreational water quality qPCR methods (6-7).

The effect of DNase I treatment on growth of intact cells captured on membranes have not been examined in the literature. We found that DNase I treatment on intact cells resulted in significantly more growth of *E. coli* compared to *Ent. faecalis* during the 30 min incubation step at 37 ℃. A study that tested DNase I treatment on membrane filters with pure cultures did not report the same observation of growth (48). However, conditions were different: (i) *Salmonella enterica* was the subject, (ii) solutions tested in the other study comprised of intact cells, dead bacteria and exDNA, and (iii) bacteria were exposed to a high temperature DNase inactivation step of 54 ℃ for 1 hour (48). Our study did not include a DNase inactivation step, but this protocol should be explored in future experiments.

Recycled water is one major source of exDNA that can enter environmental waters by direct discharge or by stormwater runoff. Few studies have tested the ability to differentiate the qPCR signal of DNA from intact cells and exDNA in recycled water. When we filtered river water and recycled water with spiked exDNA derived from lysed cells of *N. pharaonis*, an exogenous archaeon, the DNA marker NP*gyr*A captured on membranes was significantly reduced by DNase I treatment but remained quantifiable in all samples. DNase efficacy may have been reduced in river water and recycled water due to environmental variables (e.g., antibiotics or inorganic

substances) that could have formed stable complexes with DNA and inhibited DNase activity (55). On the other hand, one study found that GC rich DNA, as found in the genome of *N. pharaonis* (56), can lead to poor efficacy of DNase cleavage (57). The genomes of bacteria such as *E. coli* and *Ent. faecalis* have a much lower GC than *N. pharaonis* DNA (57-59); therefore, DNase efficacy may have been dependent on the organism used to lyse and release exDNA that was then spiked into river water and recycled water. Although we did not completely eliminate exDNA from river water or recycled water on membranes, we were able to reduce levels by 100-fold. The null hypothesis of no effect of DNase I treatment was rejected. Complete digestion of exDNA from recycled water and river water captured on membranes may be achieved with an increased dose of DNase as well as longer exposure times.

There are no examples in the literature for the use of DNase I treatment in recycled water samples, but there are a few studies that have attempted to differentiate intact cells from extracellular DNA. One study on chlorine-disinfected secondary effluent from a wastewater treatment facility in Ohio, U.S. used PMA treatment prior to qPCR and found that roughly 93% of the *Enterococcus* qPCR signal was from exDNA (60). In contrast, we estimated by DNase I treatment that approximately 98% of the HF183 qPCR signal captured on polycarbonate membranes following filtration of recycled water was derived from exDNA. However, the study in Ohio avoided membrane filtration as the authors noted that washing steps resulted in a significant loss of intact cells by this treatment method, and sample sizes were limited to small volumes (i.e., 1 mL) to avoid an overestimation of intact cells (60). Other studies that have utilized this approach have found similar limitations with larger volumes (≥ 10 mL) of disinfected wastewater effluent, where suspended solids interfered with PMA efficiency (61-62). Furthermore, recreational water quality studies generally require filtration of large volumes (500

mL – 2 L) to detect DNA markers in surface waters (9-13, 35), which highlights the advantage of using DNase I treatment over PMA. Further analysis of recycled water from different wastewater treatment facilities by DNase I treatment coupled with microscopy or flow cytometry will improve our understanding on the extent to which this source of exDNA, that may enter surface waters in large volumes, could interfere with studies focused on recreational water quality.

Conclusion

- Roughly 1% of exDNA from pure cultures of gram-positive *Ent. faecalis* and gram-negative *E. coli* was captured on polycarbonate membranes and measured by qPCR (Entero1A and EC23S857) following standard recreational water quality methods.
- River water and recycled water spiked with exDNA from a pure culture of *N. pharaonis* showed that the percentage of qPCR gene copies (NP*gyr*A) captured on polycarbonate membranes was consistent and ranged from ~ 1 – 2%.
- DNase I effectiveness was demonstrated on extracellular DNA derived from pure cultures and complete digestion was found on membrane filters for Entero1A and EC23S857; however, DNase I treatment only digested most of the extracellular DNA in recycled water and river water.
- DNase I treatment of membrane filters represents a potentially useful path to significantly mitigate qPCR interference from exDNA captured by recreational water quality techniques and may be improved by increased enzyme concentrations or exposure times.

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process. We also thank John Griffith and the Southern California Coastal Water Research Project for providing the *N. pharaonis* strain and protocol for cultivation and qPCR.

Table 4.1. Primer and probe sequences and qPCR cycling parameters for assays used.

DNA Markers	Primer/Probe sequences (5'-3')	Cycling parameters	Citation
EC23S857	forward primer (1 µM): GGTAGAGCACTGTTTTGGCA reverse primer (1 µM): TGTCTCCCGTGATAACTTTCTC probe (80 nM): FAM-TCATCCCGACTTACCAACCCG-TAMRA	10 min at 95 °C followed by 40 cycles of (15 s at 95 °C and 60 s at 56 °C)	(47)
Entero1A	forward primer (1 µM): GAGAAATTCCAAACGAACTTG reverse primer (1 µM): CAGTGCTCTACCTCCATCATT probe (80 nM): [6~FAM]TGGTTCTCTCCGAAATAGCT[TAMRA~Q]	2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of (15 s at 95 °C, 60 s at 60 °C)	(4)
NP*gyr*A	forward primer (0.5 µM): ACGATTACCTGCTCTGCTTTAC reverse primer (0.5 µM): CGTTGAGGTCGAGAACATTGA probe (80 nM): [FAM]-CAAGGGCAGGTCTATCGGCTGAAG-[BHQ1]	2 min at 50 °C, 10 min at 95 °C followed by 40 cycles of (15 s at 95 °C and 60 s at 60 °C)	(32)
HF183/ BacR287	forward primer (1 µM): ATCATGAGTTCACATGTCCG reverse primer (1 µM): CTTCCTCTCAGAACCCCTATCC BacP234MGB (80 nM): [6-FAM]-CTAATGGAACGCATCCC-MGB	2 min at 50 °C, 10 min at 95 °C followed by 40 cycles of (15 s at 95 °C and 60 s at 60 °C)	(5)

Table 4.2. Average of total gene copies (mean percentage captured) measured in 1 mL from each sample type by qPCR (n=6) for pure cultures experiments with *E. coli* (EC23S857) and *Ent. faecalis* (Entero1A).

DNA Markers	Intact Cells			exDNA		
	Filtrate	Membrane	Membrane + DNase I	Filtrate	Membrane	Membrane + DNase I
EC23S857	3.24 (0.4)	5.61 (85.6)	5.90 (169.5)	5.54 (55.8)	3.92 (1.4)	< LOD[a]
Entero1A	3.15 (1.2)	5.00 (80.0)	5.13 (108.0)	5.45 (50.8)	3.36 (0.5)	< LOD

[a] < LOD = below limit of detection.

Table 4.3. Differences of gene copies captured on membranes between *E. coli* (EC23S857) and *Ent. faecalis* (Entero1A) intact cells and exDNA, and the effect of DNase I treatment on filter membranes tested by ANOVA (n=6). P-values < 0.05 are bolded.

Statistical Tests	Intact Cells	exDNA
EC23S857 vs. Entero1A captured on membrane	0.0793	**0.0001**
EC23S857 on membrane vs. membrane + DNase I	**< 0.0001**	**< 0.0001**
Entero1A on membrane vs. membrane + DNase I	**< 0.0001**	**0.0005**

Table 4.4. Average of total gene copies (mean percentage captured) measured in 1 mL from each sample type (n=6) by qPCR for river water and recycled water spiked with exDNA (NP*gyr*A) derived from *N. pharaonis* culture. HF183 was not spiked into samples and percentage captured was not calculated for this DNA marker but gene copies measured was also examined for each sample type.

DNA Markers	River Water			Recycled Water		
	Filtrate	Membrane	Membrane + DNase I	Filtrate	Membrane	Membrane + DNase I
NP*gyr*A	5.48 (44.23)	3.62 (0.62)	2.56 (0.06)	5.39 (35.71)	3.95 (1.32)	2.35 (0.03)
HF183	< LOD[a]	< LOD	< LOD	3.11	4.42	2.71

[a] < LOD = below limit of detection.

Table 4.5. Differences of gene copies captured on membranes from spiked river water or recycled water, and the effect of DNase I treatment on filter membranes tested by ANOVA (n=6). P-values < 0.05 are bolded.

Statistical Tests	River Water	Recycled Water
NP*gyr*A on membrane with and without DNase treatment	**< 0.0001**	**< 0.0001**
HF183 on membrane with and without DNase treatment	NA[a]	**< 0.0001**

[a] NA = HF183 was not detected in river water, samples were not included for statistical analysis.

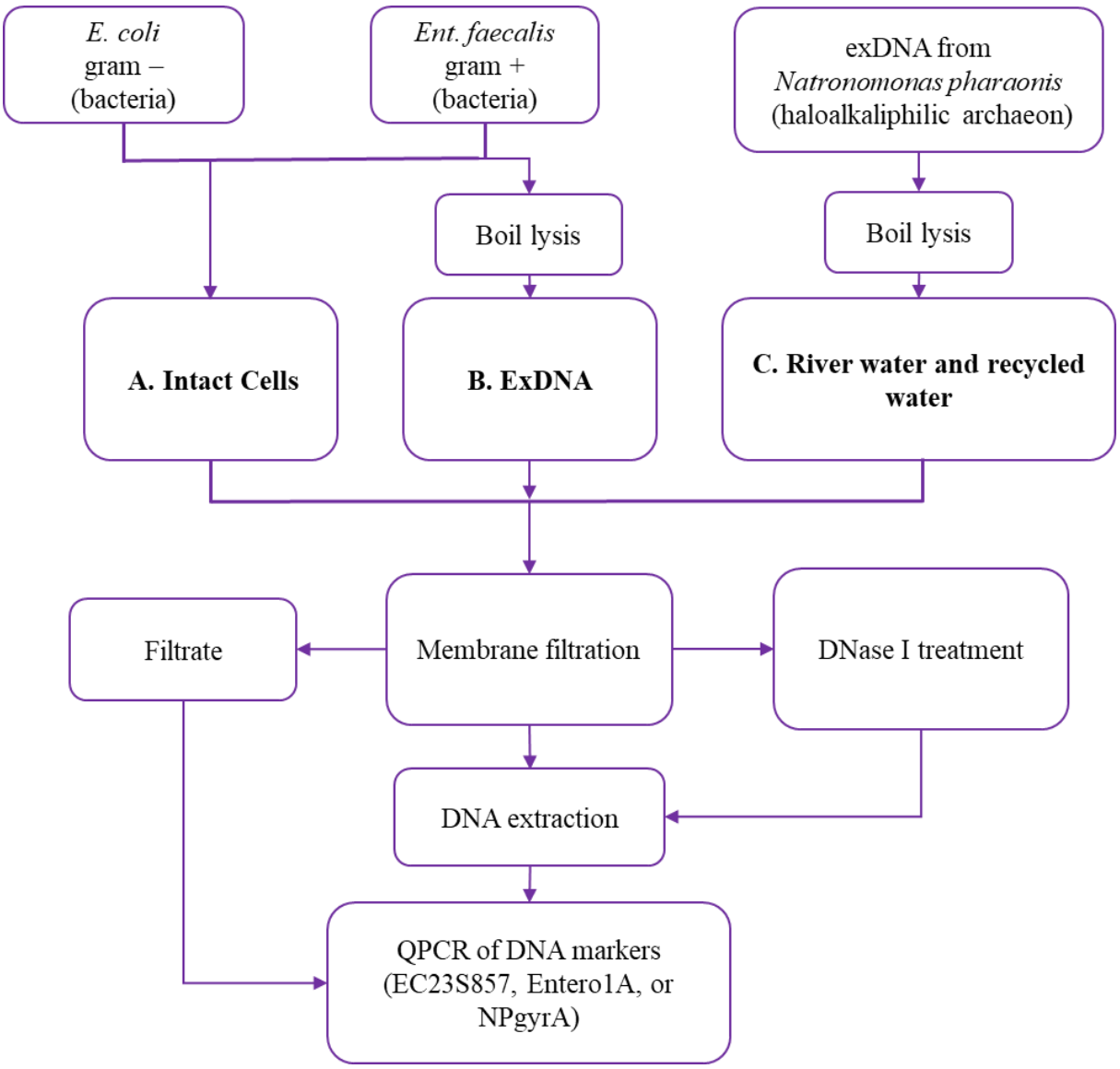

Figure 4.1. Experimental design for comparing the qPCR signal captured on membrane filters from intact cells and exDNA from *E. coli* and *Ent. faecalis*, exDNA from *N. pharaonis* in river water and recycled water, and the effect of DNase I treatment on membrane filters.

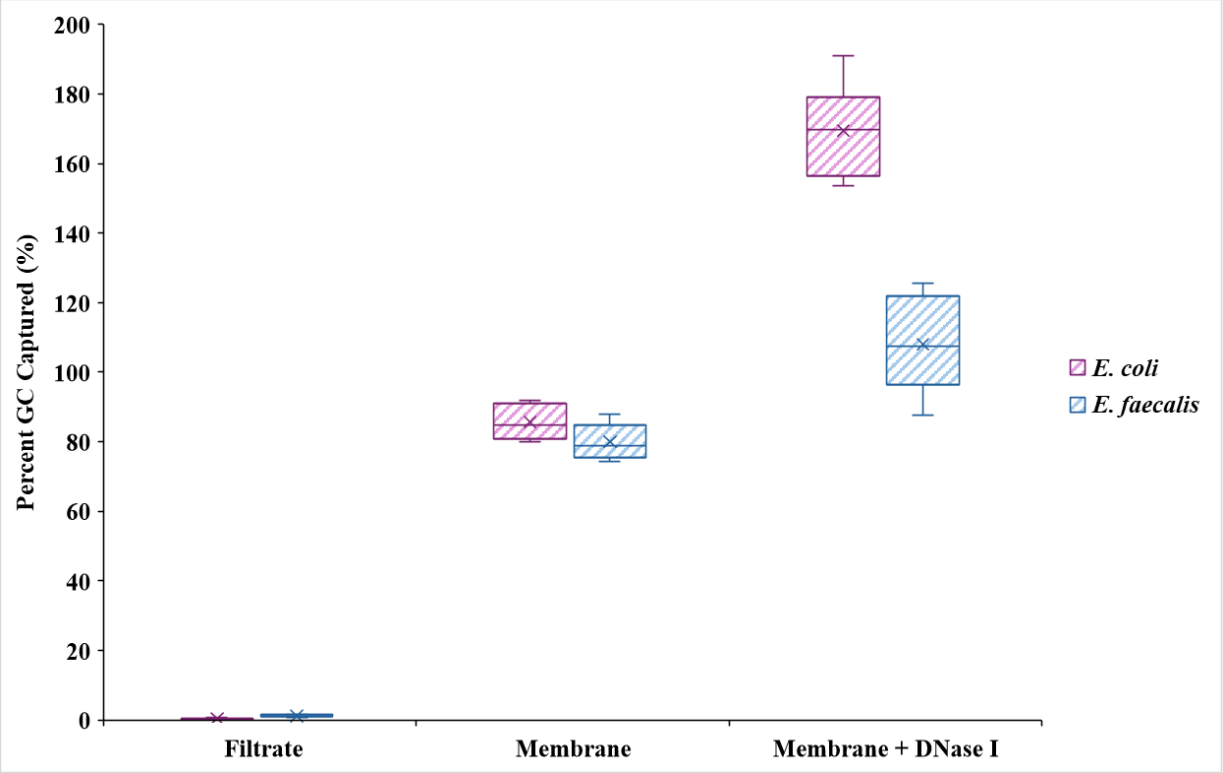

Figure 4.2. The percentage of gene copies (GC) from intact cells of *E. coli* (pink) and *Ent. faecalis* (blue) in filtrate following membrane filtration, and on the filter membrane without and with DNase I treatment. DNA of microbial targets was measured by qPCR (EC23S857 and Entero1A). The interquartile range (25^{th} and 75^{th} percentile) includes the $\log_{10}$ medians (horizontal bar) and means (X) for DNA marker. Boxplot whiskers represent the 10^{th} and 90^{th} percentile values.

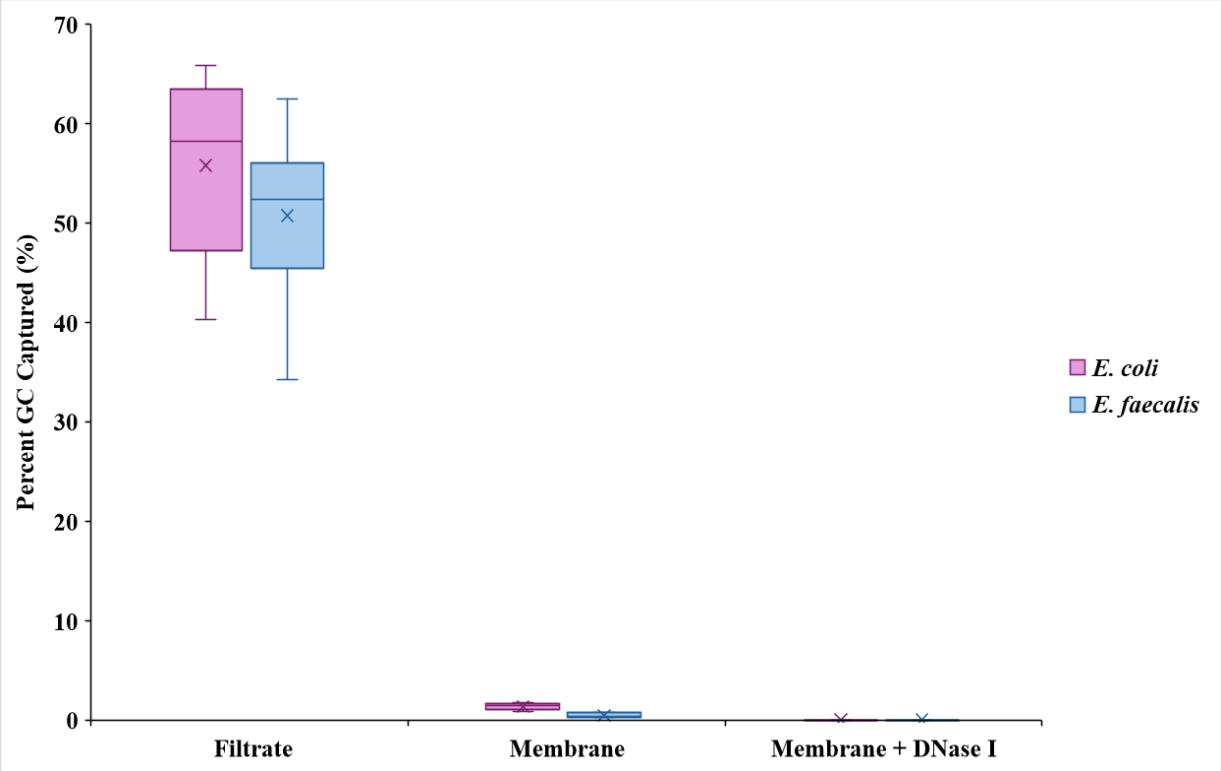

Figure 4.3. Percent of gene copies (GC) from lysed cells of *E. coli* (pink) and *Ent. faecalis* (blue), exDNA captured in filtrate, and on the filter membrane without and with DNase I treatment. DNA of microbial targets was measured by qPCR (EC23S857 and Entero1A). The interquartile range (25^{th} and 75^{th} percentile) includes the $\log_{10}$ medians (horizontal bar) and means (X) for DNA marker. Boxplot whiskers represent the 10^{th} and 90^{th} percentile values.

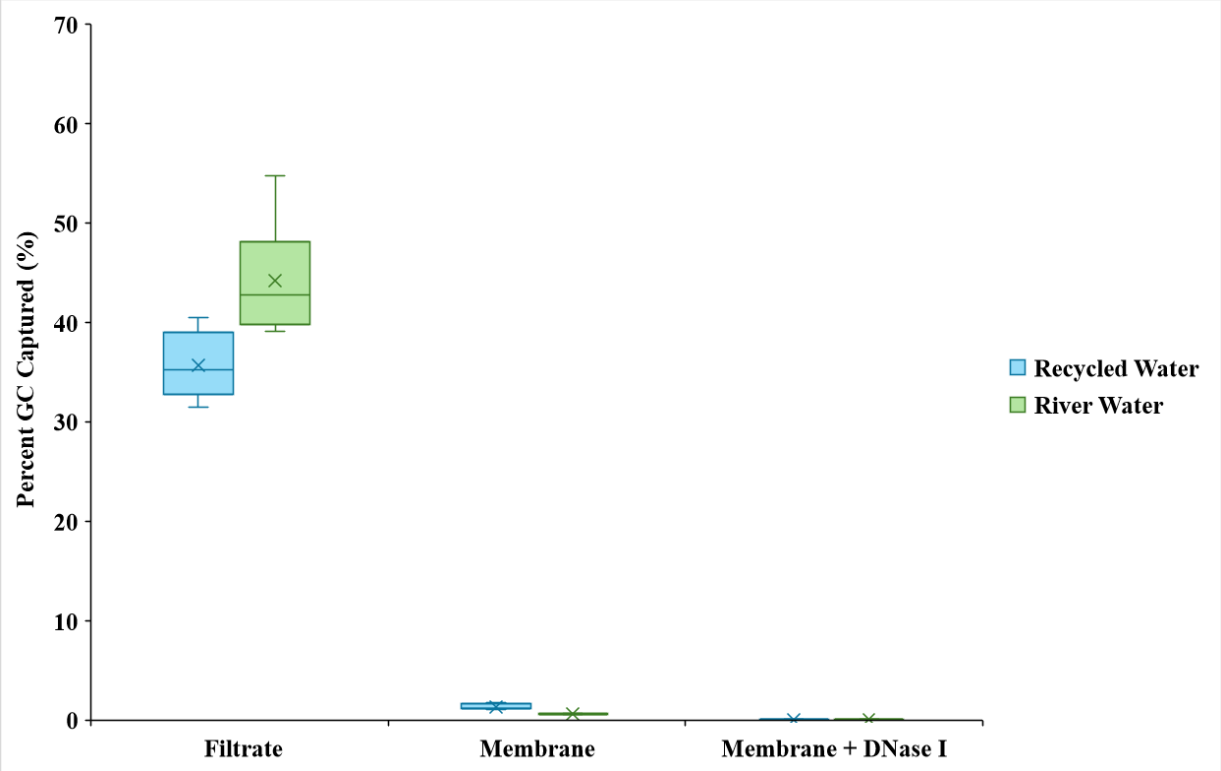

Figure 4.4. Percent of gene copies (GC) from lysed *N. pharaonis* (NP*gyr*A), exDNA spiked into recycled water (blue) or river water (green) captured in filtrate, and on the filter membrane without and with DNase I treatment. The interquartile range (25^{th} and 75^{th} percentile) includes the $\log_{10}$ medians (horizontal bar) and means (X) for DNA marker. Boxplot whiskers represent the 10^{th} and 90^{th} percentile values.

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AFTERWORD

Summary

This research has expanded the field of recreational water quality including MST by i) demonstrating the persistence of DNA markers through different levels of wastewater treatment and providing a tool to help distinguish untreated sewage from recycled water in surface waters, ii) confirming that DNA markers from recycled water can persist longer than DNA from untreated sewage while exposed to environmental conditions, and iii) establishing that extracellular DNA can be captured by standard recreational water quality methods, while DNase I treatment has potential to eliminate undesirable extracellular DNA from concentrated environmental water samples.

My research has shown that there is a strong potential that recycled water can interfere with recreational water quality studies, which include microbial source tracking techniques that aim to identify untreated sewage. We demonstrated an approach with culturable EcH8 to confirm the presence of untreated sewage while avoiding DNA from recycled water or the environment. My findings also support the use of an enzyme treatment (DNase I) to reduce the amount of extracellular DNA detected while using qPCR-based recreational water quality techniques.

Future Directions

This research has shown the usefulness of two different approaches to circumvent interference from extracellular DNA captured by standard recreational water quality methods. However, more investigations will be needed with culturable H8 measure its persistence in different

environments (e.g., marine water and direct sunlight) and to further examine this markers ability to confirm sewage in surface waters across geographic locations. Furthermore, this method is limited by the number of colonies tested and would benefit from testing larger composites of *E. coli*. For the DNase I approach, future experiments should be done on intact cells isolated from the environment and exploring the effects of increased doses and exposure times to eliminate extracellular DNA from environmental water. These steps will expand our understanding of the extent to which each approach can be applied to improve estimations of human health risk in recreational water quality studies.

APPENDIX A: CHAPTER TWO (COPYRIGHT PERMISSON)

Chapter two of the dissertation was previously published, entitled: Persistence of Sewage-Associated Genetic Markers in Advanced and Conventional Treated Recycled Water: Implications for Microbial Source Tracking in Surface Waters. Published in *mBio* (2024) DOI: 10.1128/mbio.00655-24 and has been reprinted with permission from *mBio*: "ASM grants authors the right to republish discrete portions of their article in any other publication (including print, CD-ROM, and other electronic formats), provided that proper credit is given to the original ASM publication. ASM authors also retain the right to reuse the full article in their dissertation or thesis".

APPENDIX B: CHAPTER TWO SUPPLEMENTARY MATERIALS

Table B.1. Primer and probe concentrations and sequences, qPCR cycling parameters for assays used.

QPCR Targets	Primer/Probe sequences (Final concentration): 5'-3'	Cycling parameters	Citation
H8	forward primer (0.9 µM): ACAGTCAGCGAGATTCTTC reverse primer (0.9 µM): GAACGTCAGCACCACCAA probe (80 nM): FAM-ACTGGCATCGGCATGGAACAC-BHQ	2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of (15 s at 95 °C, 60 s at 58 °C)	(35)
*uid*A	forward primer (1 µM): CAACGAACTGAACTGGCAGA reverse primer (1 µM): CATTACGCTGCGATGGAT probe (80 nM): VIC-CCCGCCGGGAATGGTGATTAC	2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of (15 s at 95 °C, 60 s at 60 °C)	(39)
EC23S857	forward primer (1 µM): GGTAGAGCACTGTTTTGGCA reverse primer (1 µM): TGTCTCCCGTGATAACTTTCTC probe (80 nM): FAM-TCATCCCGACTTACCAACCCG-TAMRA	10 min at 95 °C followed by 40 cycles of (15 s at 95 °C and 60 s at 56 °C)	(37)
HF183/ BacR287	forward primer (1 µM): ATCATGAGTTCACATGTCCG reverse primer (1 µM): CTTCCTCTCAGAACCCCTATCC BacP234MGB (80 nM): FAM-CTAATGGAACGCATCCC-MGB	2 min at 50 °C, 10 min at 95 °C followed by 40 cycles of (15 s at 95 °C and 60 s at 60 °C)	(7)
CrAssphage CPQ-056	forward primer (1 µM): CAGAAGTACAAACTCCTAAAAAACGTAGAG reverse primer (1 µM): GATGACCAATAAACAAGCCATTAGC probe (80 nM): FAM-AATAACGATTTACGTGATGTAAC-MGB	10 min at 95°C followed by 40 cycles of (15 s at 95 °C and 1 min at 60 °C)	(38)

Table B.2. QPCR assay amplicon length and sequences utilized in gBlocks™ material for standard curves.

Assay	Amplicon length of qPCR target (bp)	Gene sequence (5'-3')
H8	177	ACAGTCAGCGAGATTCTTCGCCACGCCGGCGTGGCG CATCTGCTGCTGGAGGCGGACGCGCAGAAGGTCGAG GCCGCGCGTGCCGCCGGCGCGCCGGTGTTCCATGCC GATGCCAGTCGGCCCGATACCTTGCTGGCTGCCGGC TTGACGCATGCACACTTGGTGGTGCTGACGTTC
EC23S857	88	GGTAGAGCACTGTTTTGGCAAGGGGGTCATCCCGAC TTACCAACCCGACTCGAGCTGCGAATACCGGAGAAA GTTATCACGGGAGACA
HF183/ BacR287	132	ATCATGAGTTCACATGTCCGCATGATTAAAGGTATTT TCCGGTAGACGATGGGGATGCGTTCCATTAGCTCGA GATAGTAGGCGGGGTAACGGCCCACCTAGTCAACGA TGGATAGGGGTTCTGAGAGGAAG
CrAssphage CPQ-056	126	CAGAAGTACAAACTCCTAAAAAACGTAGAGGTAGA GGTATTAATAACGATTTACGTGATGTAACTCGTAAA AAGTTTGATGAACGTACTGATTGCAACAAAGCTAAT GGCTTGTTTATTGGTCATC

Table B.3. Statistical comparisons of microbial variables measured by qPCR in untreated sewage from AWT and CWT facilities. Data are expressed as concentration (log_{10} GC/100 mL). Data from like facilities (AWT or CWT) were pooled (n=9). Variables were individually compared between AWT and CWT facilities by Dunn rank sum tests with Bonferroni correction. Differences in frequency of detection for culturable EcH8 were compared by Fisher's Exact test. P-values < 0.05 are bolded.

Microbial Variables	**P value: AWT vs CWT**
Culturable *E. coli*	0.2670
Culturable EcH8	0.1033
EC23S857	**0.0013**
HF183	0.1440
H8	0.0849
CPQ_056	**0.0017**

Table B.4. Log_{10} reduction values (mean ± standard error) of all qPCR targets in conventional (CWT) and advanced (AWT) wastewater treatment facilities (n=3).

Conventional	**EC23S857**	**HF183**	**H8**	**CPQ_056**
A	4.67 ± 0.64	4.01 ± 0.99	4.35 ± 0.96	1.72 ±0.31
B	4.70 ± 1.25	4.55 ± 1.31	4.32 ± 1.09	1.93 ± 0.07
C	5.77 ± 0.03	5.58 ± 0.18	5.57 ± 0.25	1.82 ± 0.20
Advanced				
D	4.75 ± 0.57	4.12 ±0.13	3.74 ± 0.36	5.17 ± 0.35
E	6.61 ± 0.39	6.42 ± 0.63	6.08 ± 0.16	6.82 ± 0.12
F	5.00 ± 1.28	6.85 ± 0.16	5.91 ± 0.05	4.50 ± 1.20

Table B.5. *P* values for statistical comparisons among microbial variables measured by qPCR in pooled untreated sewage and recycled water data (n=18). P-values < 0.05 are bolded.

qPCR Variables	Untreated Sewage	Recycled Water		
	Median log_{10} concentration	**Median log10 concentration**	**Frequency of detection**	**Log_{10} reduction**
EC23S857 : HF183	0.0512	0.3390	**0.0455**	> 0.9999
EC23S857 : H8 marker	**< 0.0001**	0.0765	**0.0191**	> 0.9999
EC23S857 : CPQ_056	**0.0021**	0.3170	0.1040	0.0832
H8 marker : HF183	**< 0.0001**	0.2610	1.0000	> 0.9999
H8 marker : CPQ_056	**0.0017**	**0.0084**	0.7110	0.3740
HF183 : CPQ_056	0.2430	0.0744	1.0000	0.1280

Table B.6. Significant relationships among concentrations of microbial variables in untreated sewage and recycled water pooled data (n=18). Kendall's tau reflects the ordinal association of the data; higher values indicate greater correlation. P-values < 0.05 are bolded.

qPCR Variable 1	qPCR Variable 2	Untreated Sewage		Recycled Water	
		p-value	tau	p-value	tau
EC23S857	HF183	0.1751	0.24	**0.0005**	0.62
EC23S857	H8 marker	**0.0006**	0.57	**0.0136**	0.44
EC23S857	CPQ_056	**0.0067**	0.46	0.2690	0.19
H8 marker	HF183	**0.0022**	0.52	**0.0001**	0.74
H8 marker	CPQ_056	**0.0085**	0.45	0.1062	0.29
HF183	CPQ_056	0.0573	0.33	0.0760	0.32

Table B.7. Surface water survey and the frequency of detection for HF183 and culturable EcH8 for each site. All samples were collected monthly between 8/11/2020 and 8/9/2022.

Surface Water Sites	Water Type	Number of samples tested	HF183 frequency of detection (%)	EcH8 frequency of detection (%)
S1	Freshwater	16	68.75	6.25
S2	Estuarine	17	100	11.76
S3	Estuarine	16	93.75	6.25
S4	Estuarine	8	100	0.00
S5	Freshwater	4	0.00	0.00
S6	Marine	16	81.25	12.50
S7	Freshwater	14	100	64.29
S8	Marine	12	58.33	16.67
Total		103	82.50	16.50

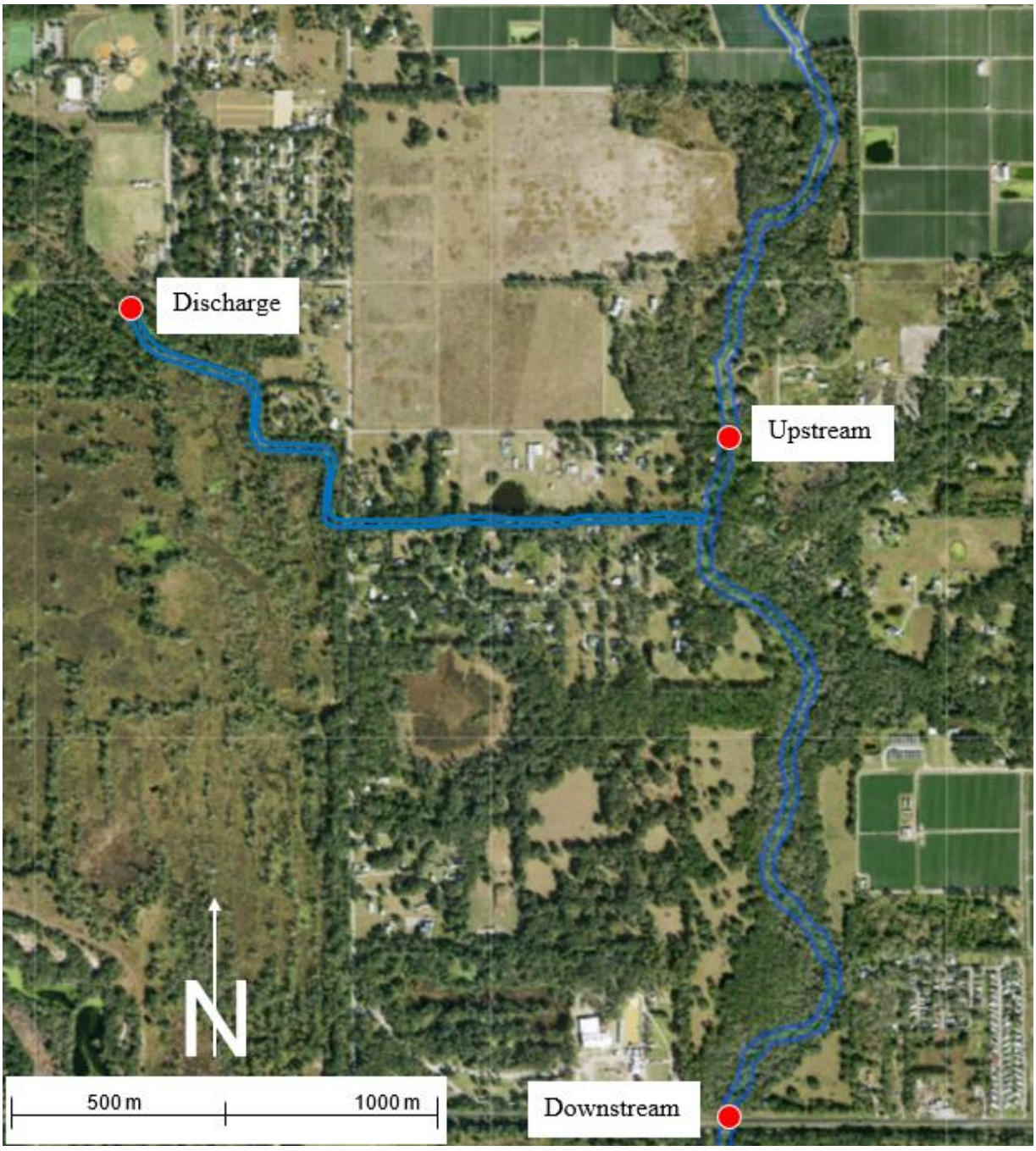

Figure B.1. Map showing the sampling locations of the field study. Recycled water travels from the discharge site along a canal to enter Turkey Creek. The length that the treated effluent travels from the discharge site to the downstream site is 3.22 km. The upstream site is 0.24 km upstream of the confluence and is not affected by the discharge. Tampa Bay Water Atlas (https://tampabay.wateratlas.usf.edu/waterbodies/rivers/74/).

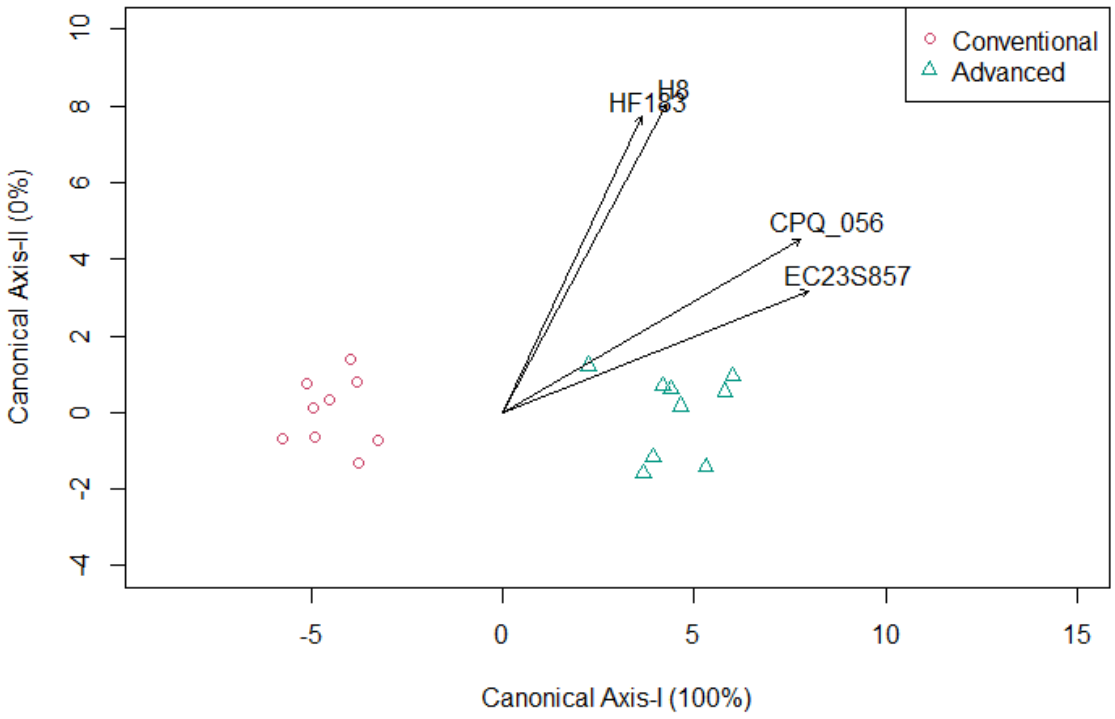

Figure B.2. Relationships among microbial variables measured by qPCR in untreated sewage collected from AWT and CWT facilities (conventional = red circles, and advanced = green triangles) analyzed by canonical analysis of principal coordinates and linear discriminant analysis. Canonical axis I (horizontal) explained 100% of the variability, while canonical axis II (vertical) explained 0% of the variability observed. Microbial variables were significantly greater in untreated sewage from AWT compared to CWT facilities ($p = 0.002$).

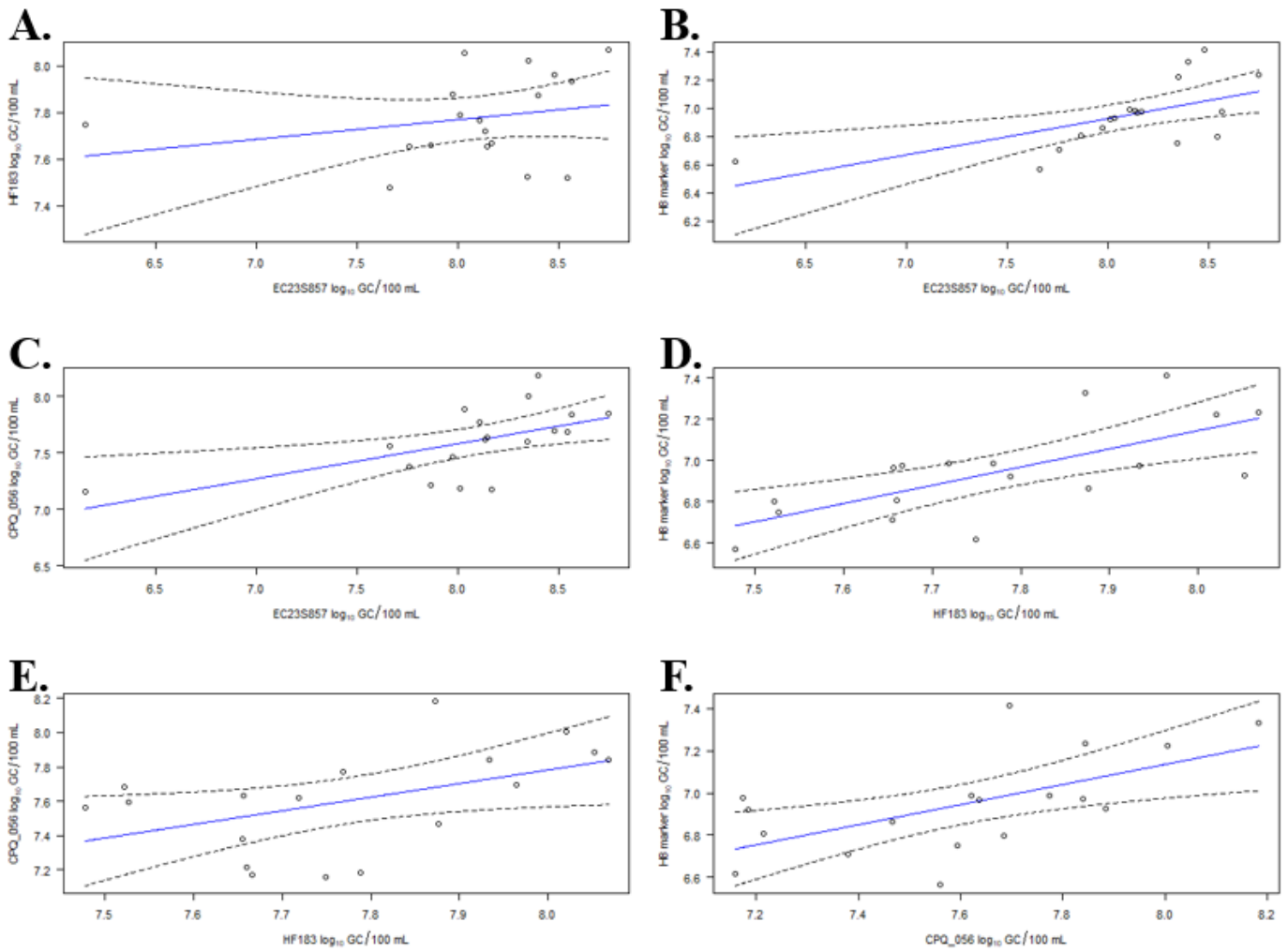

Figure B.3. Relationships among microbial variables in pooled (AWT and CWT) untreated sewage data. The solid line depicts the simple linear regression relationship (95% confidence interval shown by dashed lines). HF183 and EC23S857 (A), H8 and EC23S857 (B), CPQ_056 and EC23S857 (C), H8 and HF183 (D), CPQ_056 and HF183 (E), H8 and CPQ_056 (F).

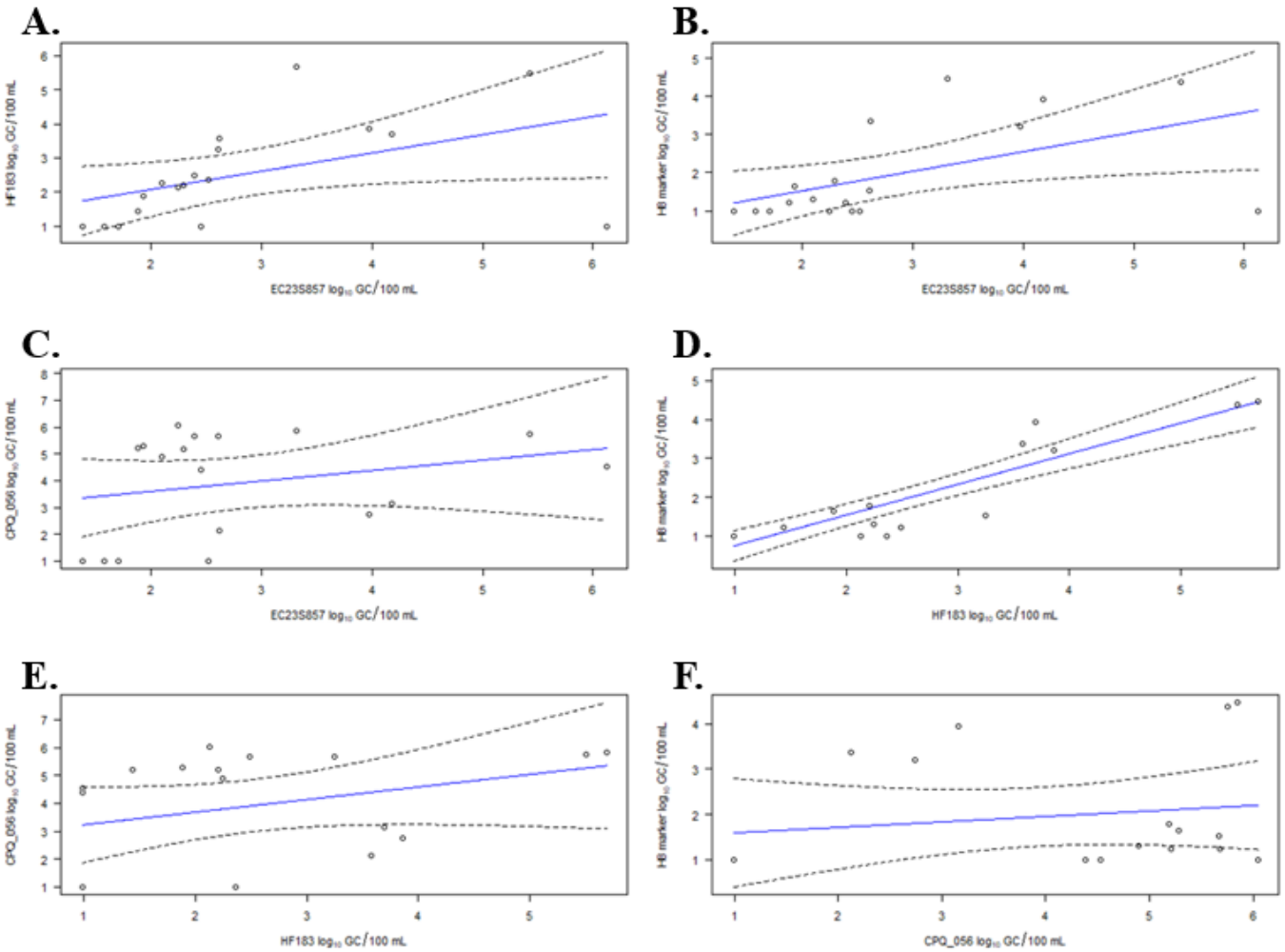

Figure B.4. Relationships among microbial variables in pooled (AWT and CWT) recycled water data. The solid line depicts the simple linear regression relationship (95% confidence interval shown by dashed lines). HF183 and EC23S857 (A), H8 and EC23S857 (B), CPQ_056 and EC23S857 (C), H8 and HF183 (D), CPQ_056 and HF183 (E), H8 and CPQ_056 (F).

APPENDIX C: CHAPTER THREE SUPPLEMENTARY MATERIALS

Table C.1. Primer and probe sequences and qPCR cycling parameters for assays used.

QPCR Targets	Primer/Probe sequences (5'-3')	Cycling parameters	Reference
H8	forward primer (0.9 µM): ACAGTCAGCGAGATTCTTC reverse primer (0.9 µM): GAACGTCAGCACCACCAA probe (80 nM): FAM-ACTGGCATCGGCATGGAACAC-BHQ	2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of (15 s at 95 °C, 60 s at 58 °C)	(21)
*uid*A	forward primer (1 µM): CAACGAACTGAACTGGCAGA reverse primer (1 µM): CATTACGCTGCGATGGAT probe (80 nM): VIC-CCCGCCGGGAATGGTGATTAC	2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of (15 s at 95 °C, 60 s at 60 °C)	(37)
EC23S857	forward primer (1 µM): GGTAGAGCACTGTTTTGGCA reverse primer (1 µM): TGTCTCCCGTGATAACTTTCTC probe (80 nM): FAM-TCATCCCGACTTACCAACCCG-TAMRA	10 min at 95 °C followed by 40 cycles of (15 s at 95 °C and 60 s at 56 °C)	(36)
HF183/ BacR287	forward primer (1 µM): ATCATGAGTTCACATGTCCG reverse primer (1 µM): CTTCCTCTCAGAACCCCTATCC Bac234IAC probe (80 nM): VIC-AACACGCCGTTGCTACA-MGB BacP234MGB (80 nM): [6-FAM]-CTAATGGAACGCATCCC-MGB	2 min at 50 °C, 10 min at 95 °C followed by 40 cycles of (15 s at 95 °C and 60 s at 60 °C)	(1)
CrAssphage CPQ-056	forward primer (1 µM): CAGAAGTACAAACTCCTAAAAAACGTAGAG reverse primer (1 µM): GATGACCAATAAACAAGCCATTAGC probe (80 nM): FAM-AATAACGATTTACGTGATGTAAC-MGB	10 min at 95°C followed by 40 cycles of (15 s at 95 °C and 1 min at 60 °C)	(5)

Table C.2. QPCR assay sequences utilized in Gblock material for standard curves.

Assay	Amplicon length of qPCR target (bp)	Gene sequence (5'-3')
H8	177	ACAGTCAGCGAGATTCTTCGCCACGCCGGCGTGGCG CATCTGCTGCTGGAGGCGGACGCGCAGAAGGTCGAG GCCGCGCGTGCCGCCGGCGCGCCGGTGTTCCATGCC GATGCCAGTCGGCCCGATACCTTGCTGGCTGCCGGC TTGACGCATGCACACTTGGTGGTGCTGACGTTC
EC23S857	88	GGTAGAGCACTGTTTTGGCAAGGGGGTCATCCCGAC TTACCAACCCGACTCGAGCTGCGAATACCGGAGAAA GTTATCACGGGAGACA
HF183/ BacR287	132	ATCATGAGTTCACATGTCCGCATGATTAAAGGTATTT TCCGGTAGACGATGGGGATGCGTTCCATTAGCTCGA GATAGTAGGCGGGGTAACGGCCCACCTAGTCAACGA TGGATAGGGGTTCTGAGAGGAAG
CrAssphage CPQ-056	126	CAGAAGTACAAACTCCTAAAAAACGTAGAGGTAGA GGTATTAATAACGATTTACGTGATGTAACTCGTAAA AAGTTTGATGAACGTACTGATTGCAACAAAGCTAAT GGCTTGTTTATTGGTCATC

Table C.3. Mean (± standard deviation) concentrations (log_{10} CFU/100 mL or log_{10} GC/100 mL) of microbial variables in river water, recycled water, and untreated sewage prior to inoculation.

Microbial Variables	River Water			Recycled Water			Untreated Sewage		
	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
Culturable *E. coli*	1.47 ± 0.07	1.66 ± 0.03	1.89 ± 0.02	< LOD[a]	< LOD	< LOD	6.61 ± 0.04	6.11 ± 0.11	6.69 ± 0.02
EC23S857	2.82 ± 0.06	2.76 ± 0.21	2.69 ± 0.16	5.06 ± 0.12	4.22 ± 0.07	4.98 ± 0.05	7.97 ± 0.10	7.52 ± 0.03	7.91 ± 0.10
HF183	< LOD	< LOD	< LOD	5.14 ± 0.11	4.33 ± 0.09	4.97 ± 0.05	7.99 ± 0.11	7.69 ± 0.02	7.79 ± 0.02
CPQ_056	< LOD	< LOD	< LOD	4.70 ± 0.12	4.10 ± 0.24	4.27 ± 0.13	8.37 ± 0.29	7.39 ± 0.05	7.68 ± 0.14

[a] < LOD = below limit of detection.

Table C.4. Mean (± standard deviation) temperature and light intensity measurements in outdoor mesocosms across three experimental trials.

Experimental Trials	Environmental Variables	
	Temperature (°C)	Light Intensity (lum/ft.^{2})
Trial 1	23.2 ± 2.2	111 ± 260
Trial 2	21.3 ± 2.4	155 ± 443
Trial 3	23.2 ± 1.8	133 ± 303

Table C.5. *P* and tau values for correlation analyses between $\log_{10}$ reductions and measurements of temperature and light intensity of each DNA marker (n=15) in recycled water or untreated sewage treatments. *P*-values < 0.05 are bolded.

DNA Markers	Recycled Water Spiked River Water				Untreated Sewage Spiked River Water			
	Temperature		Light		Temperature		Light	
	p-value	tau	p-value	tau	p-value	tau	p-value	tau
EC23S857	0.4281	- 0.17	0.0644	0.40	0.7115	0.08	**0.0231**	0.49
HF183	0.1870	- 0.28	0.2000	0.30	0.4281	0.17	0.0645	0.39
CPQ_056	0.6345	-0.10	0.4281	-0.17	0.6345	- 0.10	**0.0037**	0.62

Table C.6. Effect of trial on $\log_{10}$ reduction values for each DNA marker. Post-hoc tests with *P* values for statistical analyses on pairwise comparisons (Tukey HSD) between trials in recycled water or sewage treatments (n=5). *P*-values < 0.05 are bolded.

	Recycled Water			Untreated Sewage		
Contrast	EC23S857	HF183	CPQ_056	EC23S857	HF183	CPQ_056
Trial 1 vs. Trial 2	0.9156	0.8201	0.4253	**0.0486**	0.1020	**0.0140**
Trial 1 vs. Trial 3	0.0560	**0.0222**	0.4969	**0.0112**	**0.0043**	0.0710
Trial 2 vs. Trial 3	0.1285	0.0868	0.9916	> 0.9999	0.8670	> 0.9999

Table C.7. Effect of DNA marker on log_{10} reduction values for each trial. Post-hoc tests with *P* values for pairwise comparisons (Tukey HSD) of log_{10} reductions among marker genes measured by qPCR in recycled water or sewage treatments (n=5). *P*-values < 0.05 are bolded.

DNA Markers	Recycled Water Treatment			Untreated Sewage Treatment		
	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
CPQ_056 : EC23S857	0.9999	0.6573	0.4389	**< 0.0001**	**< 0.0001**	**0.0010**
CPQ_056 : HF183	0.9972	0.7460	0.2672	0.5287	**< 0.0001**	**< 0.0001**
EC23S857 : HF183	0.9983	0.9883	0.9378	**< 0.0001**	**< 0.0001**	**< 0.0001**

Table C.8. *P* values for correlations of $\log_{10}$ reductions among microbial variables measured by qPCR in pooled recycled water or untreated spiked river water data (n=15). *P*-values < 0.05 are bolded.

Variable 1	Variable 2	Recycled Water		Untreated Sewage	
		p-value	tau	p-value	tau
Culturable *E. coli*	CPQ_056	NA[a]	NA	**0.0041**	0.54
Culturable *E. coli*	EC23S857	NA	NA	**0.0012**	0.61
Culturable *E. coli*	HF183	NA	NA	**0.0019**	0.58
CPQ_056	EC23S857	0.4351	0.16	**0.0013**	0.60
CPQ_056	HF183	0.9226	0.03	**0.0041**	0.54
EC23S857	HF183	**< 0.0001**	0.75	**< 0.0001**	0.83

[a] NA = Few detections of culturable *E. coli* which resulted in exclusion from statistical analysis.

Table C.9. Log_{10} reduction (mean ± standard error) of each microbial variable measured (n=5) in river water spiked with recycled water or untreated sewage over 5 days in the three separate trials.

Microbial Variables	Recycled Water				Untreated Sewage			
	Trial 1	Trial 2	Trial 3	Trials 1 – 3 Combined	Trial 1	Trial 2	Trial 3	Trials 1 – 3 Combined
Culturable *E. coli*	0.92 ± 0.17	0.66 ± 0.08	0.30 ± 0.20	0.63 ± 0.14	-0.40 ± 0.04	0.46 ± 0.06	1.08 ± 0.05	0.38 ± 0.16
EC23S857	0.43 ± 0.03	0.61 ± 0.12	1.51 ± 0.17	0.85 ± 0.14	-0.42 ± 0.07	0.45 ± 0.16	0.39 ± 0.03	0.14 ± 0.12
HF183	0.41 ± 0.03	0.68 ± 0.09	1.67 ± 0.24	0.92 ± 0.16	0.75 ± 0.07	2.83 ± 0.15	2.97 ± 0.04	2.18 ± 0.28
CPQ_056	0.44 ± 0.07	1.01 ± 0.55	0.95 ± 0.70	0.80 ± 0.28	0.58 ± 0.08	1.22 ± 0.20	1.00 ± 0.04	0.93 ± 0.09

Table C.10. Significant relationships among concentrations of microbial variables in river water spiked with untreated sewage or recycled water (n=15). Kendall's tau reflects the ordinal association of the data; higher values indicate greater correlation. P-values < 0.05 are bolded.

Variable 1	Variable 2	Recycled Water		Sewage	
		p-value	tau	p-value	tau
Culturable *E. coli*	EC23S857	NA[a]	NA	< **0.0001**	0.7125
Culturable *E. coli*	HF183	NA	NA	< **0.0001**	0.7720
Culturable *E. coli*	CPQ_056	NA	NA	< **0.0001**	0.6199
EC23S857	HF183	< **0.0001**	0.7495	< **0.0001**	0.7486
EC23S857	CPQ_056	< **0.0001**	0.3781	< **0.0001**	0.7820
HF183	CPQ_056	< **0.0001**	0.4786	< **0.0001**	0.6511

[a] NA = Few detections of culturable *E. coli* which resulted in exclusion from statistical analysis.

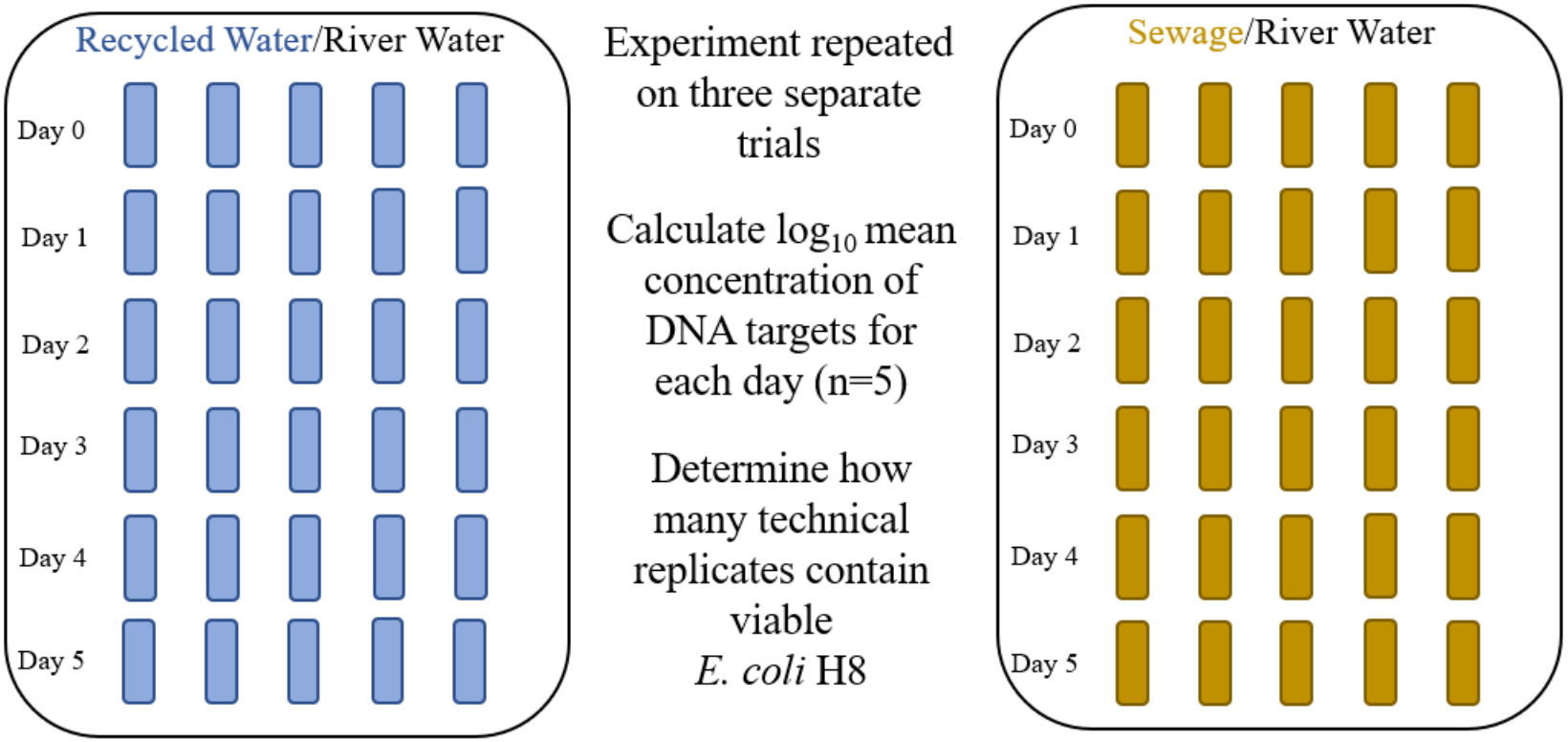

Figure C.1. Diagram of experimental design including the number of samples collected. Thirty samples were tested for each pollution type, experiment was repeated three times where 180 samples were collected and tested throughout this study.

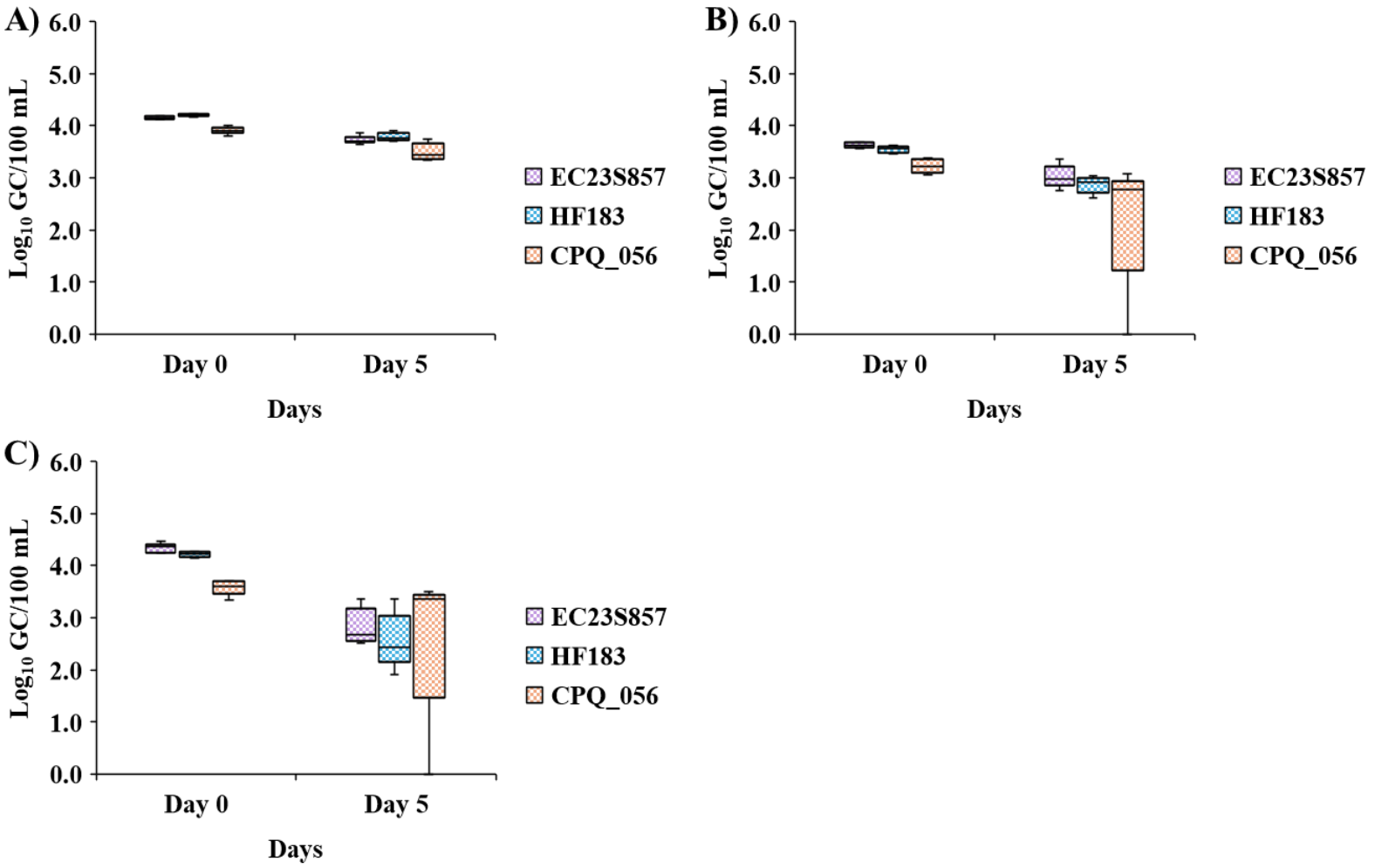

Figure C.2. Recycled water-spiked mesocosms with $\log_{10}$ concentrations (GC/100 mL) of the general *E. coli* marker EC23S857, and sewage-associated markers HF183 and CPQ_056 measured by qPCR on day 0 and day 5 for three separate events A, B, and C (n=5). Boxplot whiskers represent the 10^{th} and 90^{th} percentile values. Black bars within boxplots represent median concentrations.

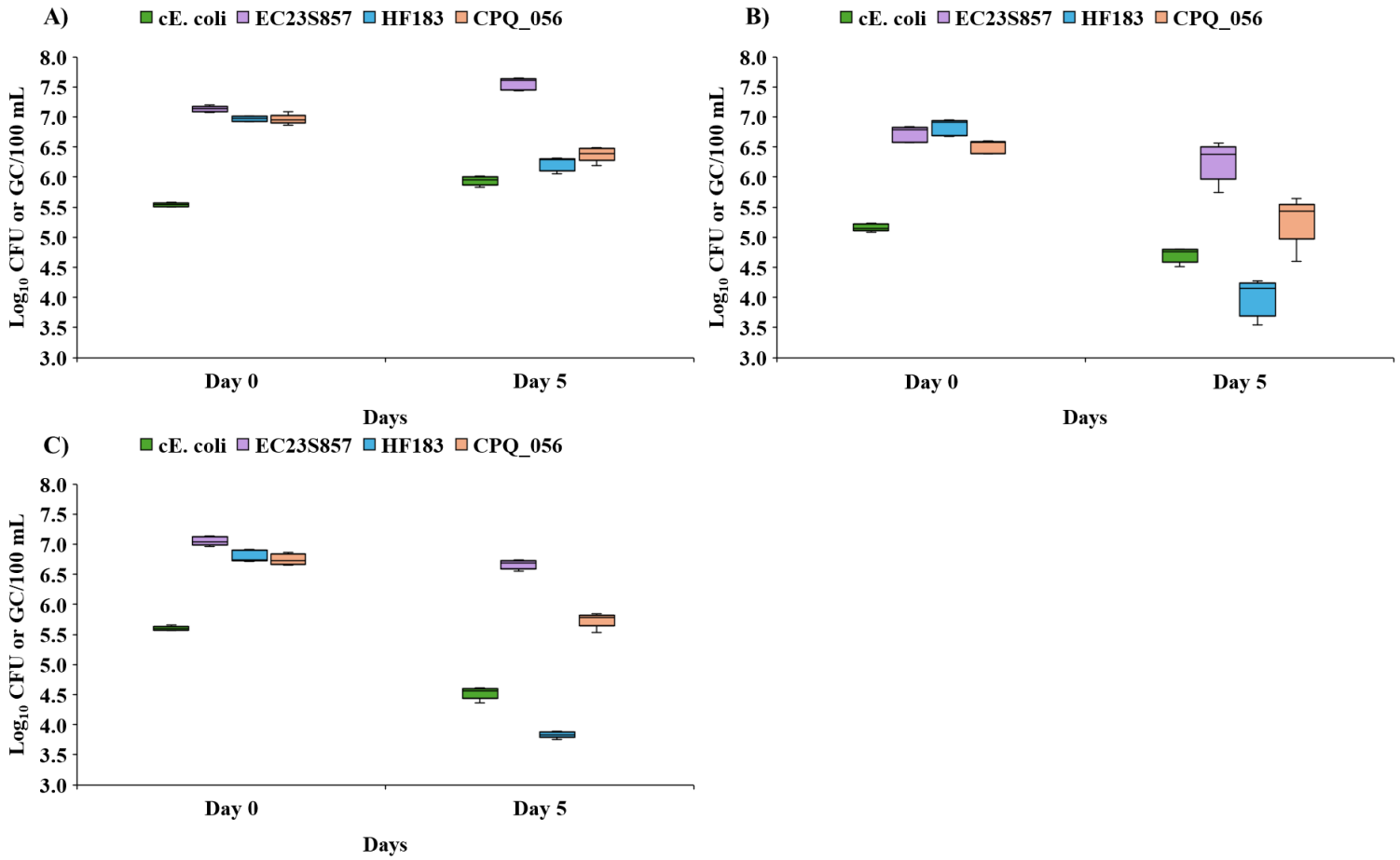

Figure C.3. Sewage-spiked mesocosms with $\log_{10}$ concentrations (CFU or GC/100 mL) of culturable *E. coli* (c*E.coli*), the general *E. coli* marker EC23S857, and sewage-associated markers HF183 and CPQ_056 measured by qPCR on day 0 and day 5 for three separate events A, B, and C (n=5). Boxplot whiskers represent the 10^{th} and 90^{th} percentile values. Black bars within boxplots represent median $\log_{10}$ concentrations, the y-axis was truncated to show data ranging from 3.0 to 8.0 $\log_{10}$ GC/100 mL.

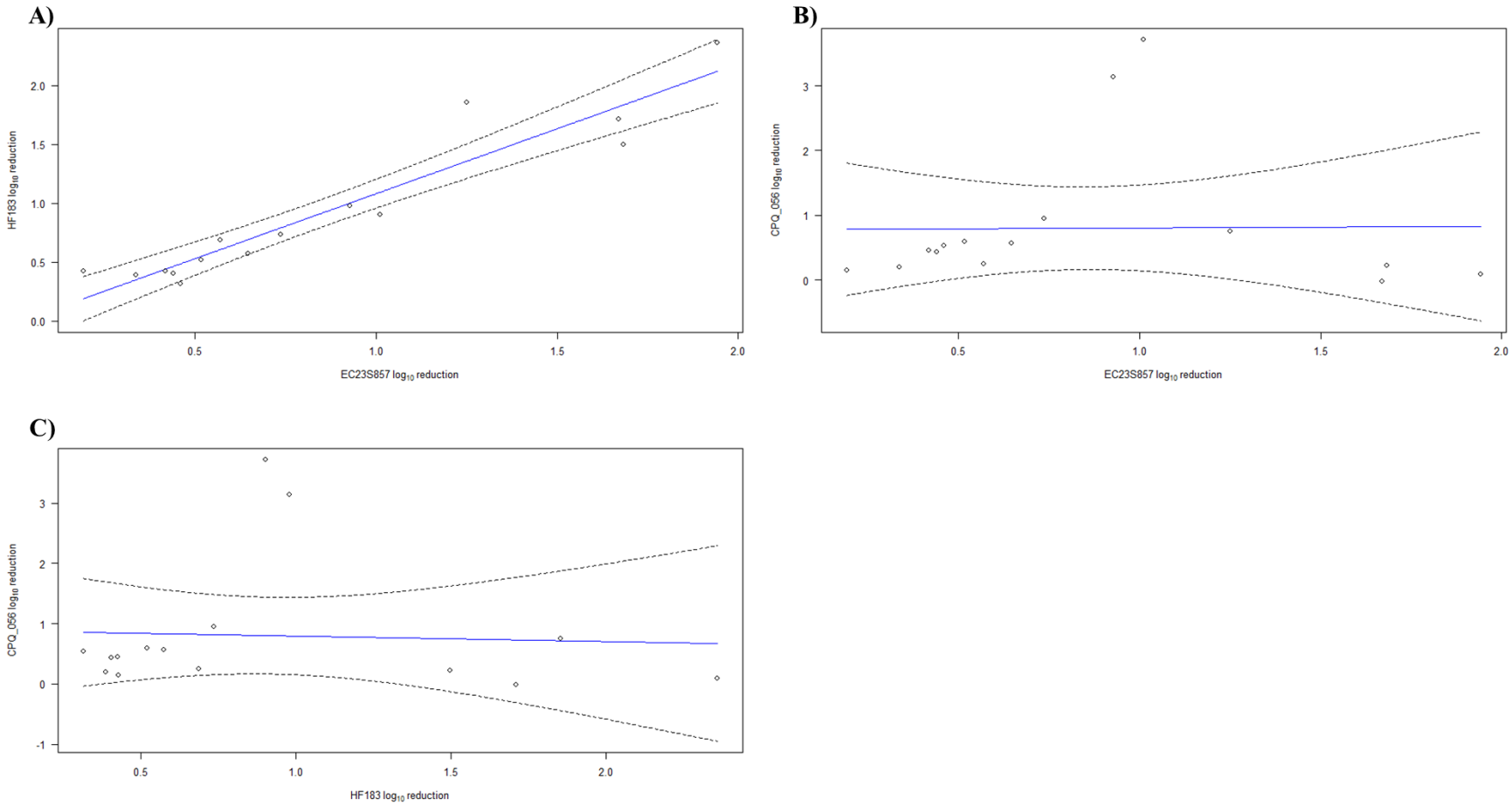

Figure C.4. Relationships among $\log_{10}$ reductions of microbial variables in river water spiked with recycled water. The solid line depicts the linear regression relationship (95% confidence interval shown by dashed lines). HF183 and EC23S857 (A), CPQ_056 and EC23S857 (B), CPQ_056 and HF183 (C).

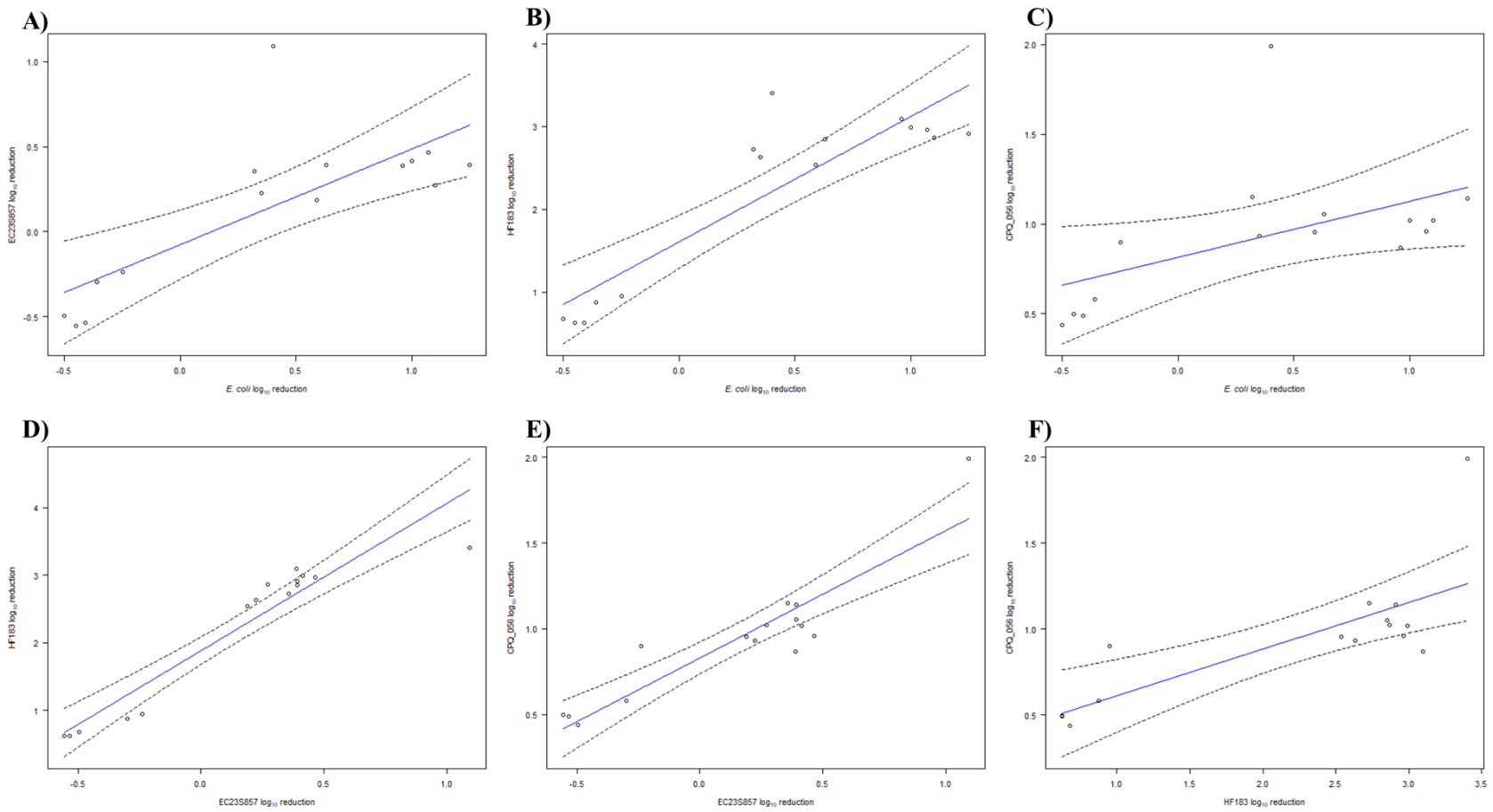

Figure C.5. Relationships among $\log_{10}$ reductions of microbial variables in river water spiked with untreated sewage. The solid line depicts the linear regression relationship (95% confidence interval shown by dashed lines). EC23S857 and culturable *E. coli* (A), HF183 and culturable *E. coli* (B), CPQ_056 and culturable *E. coli* (C), HF183 and EC23S857 (D), CPQ_056 and EC23S857 (E), CPQ_056 and HF183 (F).

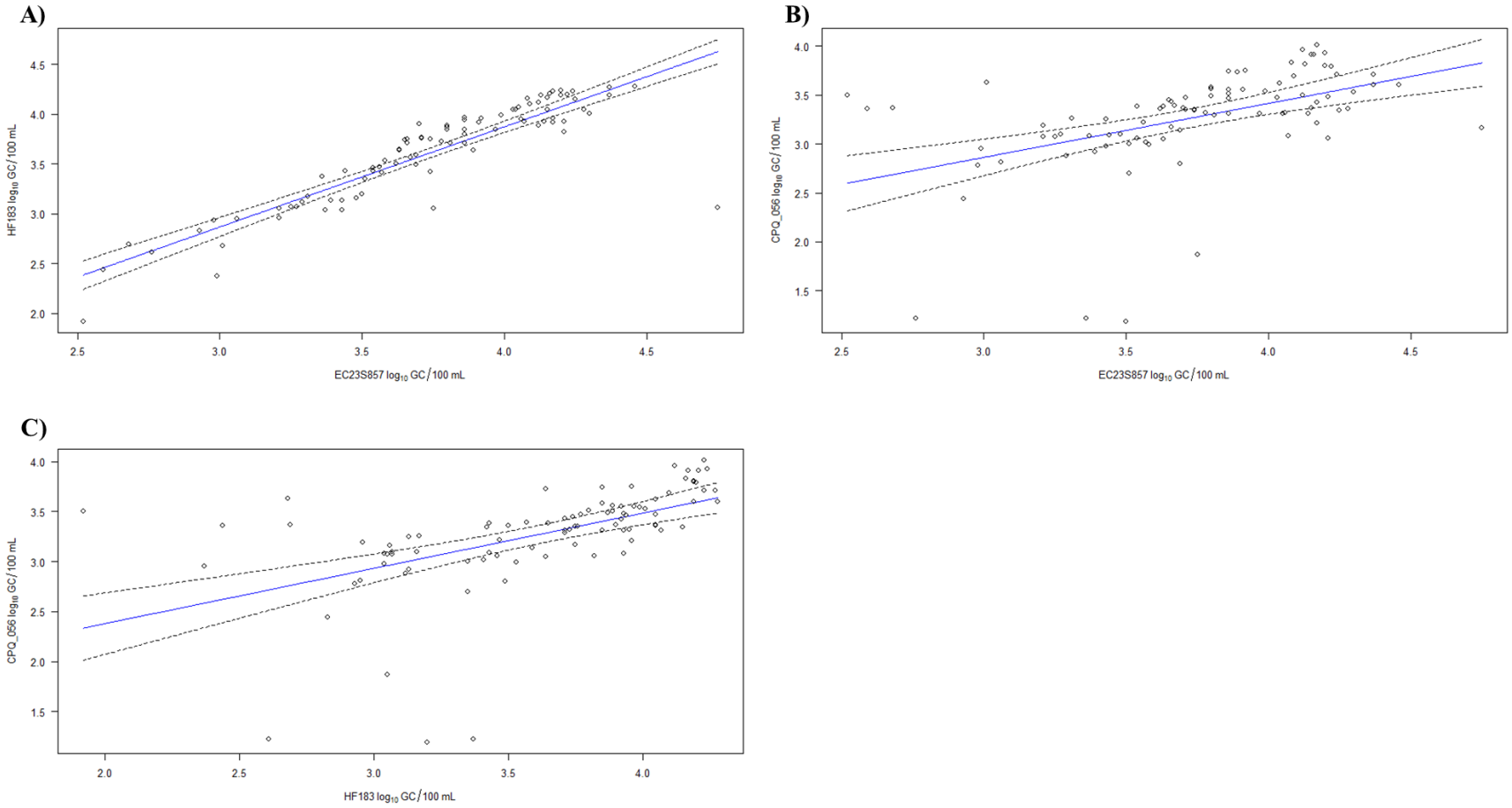

Figure C.6. Relationships among concentrations (GC/100 mL) of microbial variables in river water spiked with recycled water. The solid line depicts the linear regression relationship (95% confidence interval shown by dashed lines). HF183 and EC23S857 (A), CPQ_056 and EC23S857 (B), CPQ_056 and HF183 (C).

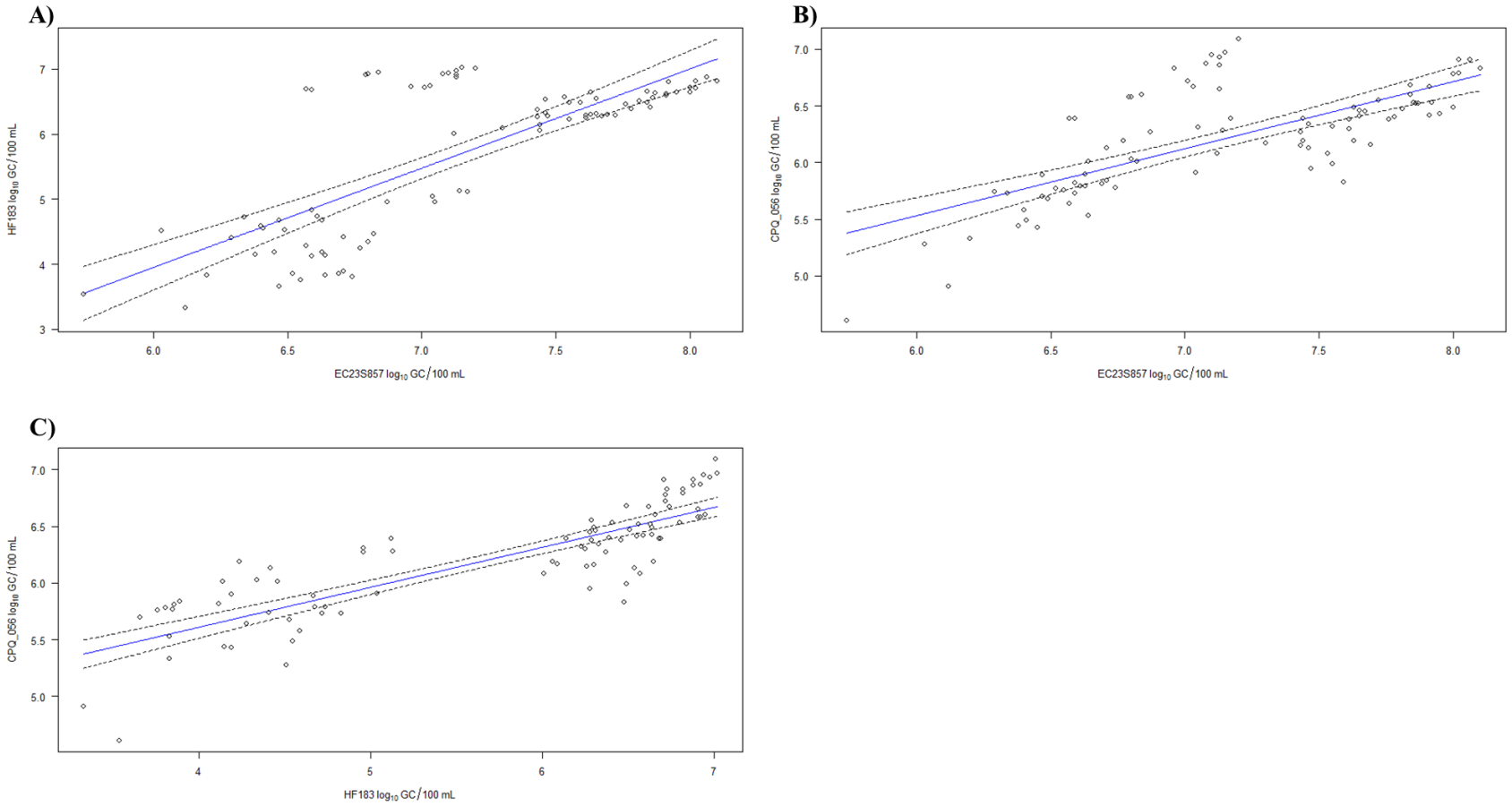

Figure C.7. Relationships among concentrations (GC/100 mL) of microbial variables in river water spiked with untreated sewage. The solid line depicts the linear regression relationship (95% confidence interval shown by dashed lines). HF183 and EC23S857 (A), CPQ_056 and EC23S857 (B), CPQ_056 and HF183 (C).

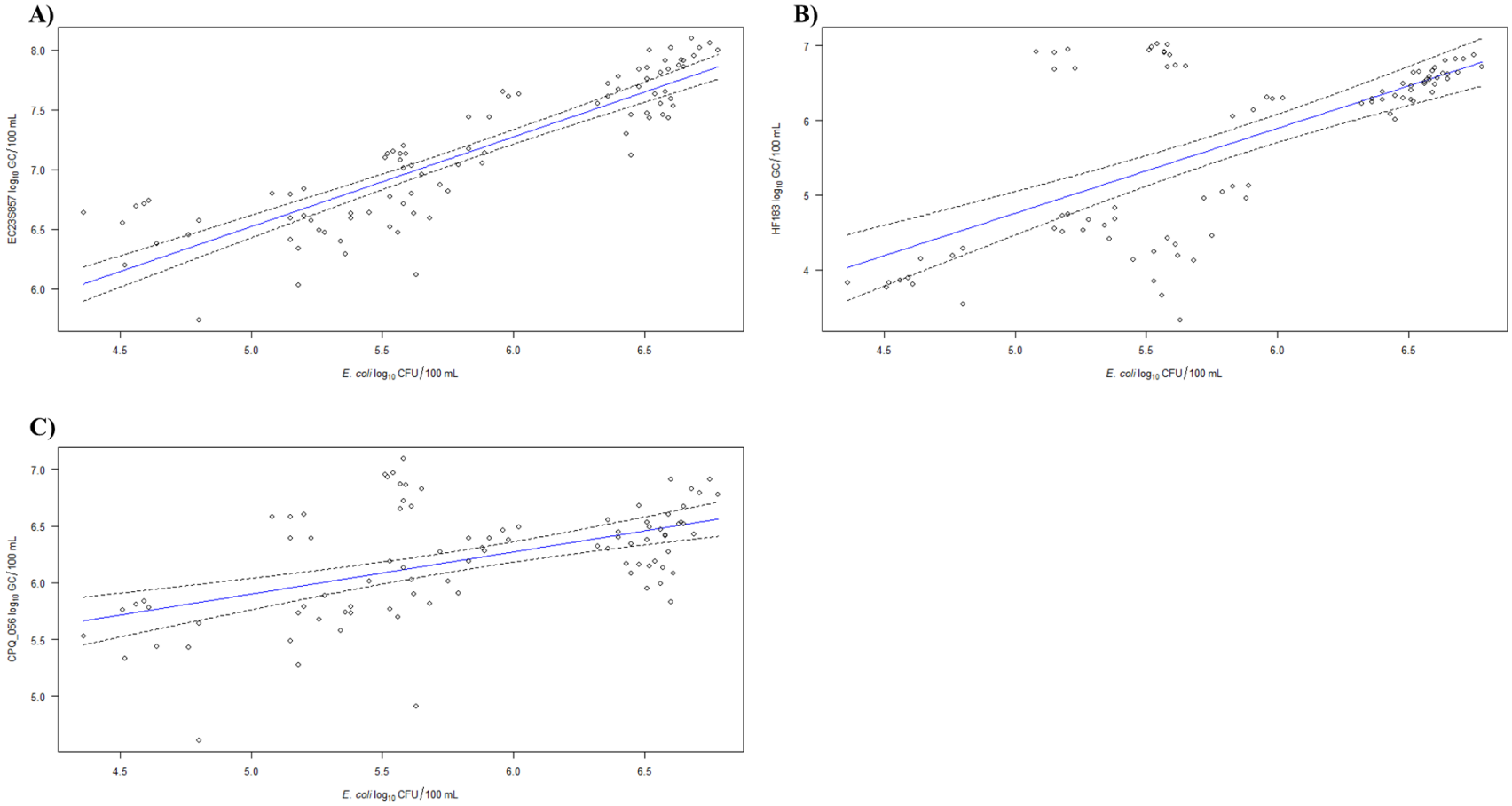

Figure C.8. Relationships among culturable *E. coli* measurements (CFU/100 mL) and $\log_{10}$ concentrations (GC/100 mL) of qPCR marker genes in river water spiked with untreated sewage. The solid line depicts the linear regression relationship (95% confidence interval shown by dashed lines). *E. coli* and EC23S857 (A), *E. coli* and HF183 (B), *E. coli* and CPQ_056 (C).