# EFFECTS OF DAIRY MANURE MANAGEMENT SYSTEMS AND WASTEWATER DISINFECTION PROCESSES ON *E. COLI* PHYLOTYPE DISTRIBUTION

A Dissertation

by

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ABSTRACT

Sections of streams located downstream from dairy farms and wastewater treatment plants (WWTPs) in central Texas are listed on the 303 (d) List of Impaired Waters due to bacterial contamination (possibly due to fecal contamination), specifically *Escherichia coli* (*E. coli*). *E. coli* can be subtyped into phylogroups that indicate potential pathogenicity; including A, B1, B2, C, D, E, F, and clades I-V; phylogroups B2 and D are considered potentially pathogenic. *E. coli* phylotype distributions were studied throughout dairy manure management systems, and before and after disinfection at WWTPs to better understand pathogenicity and reactivation of *E. coli* in downstream environments.

Four dairy farms in the Leon River Watershed of central Texas, each utilizing a different dairy manure management practice, were sampled for *E. coli* using EPA Method 1603, with a percentage of *E. coli* isolates phylotyped using the Clermont quadruplex PCR method. *E. coli* phylotypes showed no seasonal or management practice trend. B1 was the most common phylotype isolated from all dairies and time periods.

Effluent from two WWTPs (one utilizing chlorination disinfection, the other Ultra Violet (UV) light) in central Texas were sampled before and after disinfection using EPA Method 1603 for *E. coli* enumeration, and a portion of isolates were phylotyped using Clermont quadruplex PCR. *E. coli* from effluent post-disinfection was composed of higher proportions of potentially pathogenic phylotypes. A Quantitative Microbial Risk Assessment (QMRA) revealed swimming downstream from the WWTP employing UV-

irradiation may result in a slightly elevated risk of infection due to a higher portion of potentially pathogenic *E. coli*, compared to the WWTP employing chlorination.

Photoreactivation and dark repair of *E. coli* were studied in effluent from the WWTP employing UV irradiation. *E. coli* photoreactivation rates were higher than dark repair rates at 12 & 48 hours. Potentially pathogenic phylotypes represented two-thirds of all *E. coli* detected immediately after UV irradiation. However, treated effluent kept in dark conditions showed a steady decrease in potentially pathogenic phylotypes. Results from this research could help wastewater stewards lower *E. coli* concentrations and potentially pathogenic phylotype proportions in WWTP effluent.

# DEDICATION

I would like to dedicate this work to my mother, Juanita Howard. Her kindness, wisdom, faith and love gave me the strength to stand when I felt like falling.

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# NOMENCLATURE

| | |
|---|---|
| WWTPs | Wastewater Treatment Plants |
| CAFO | Confined Animal Feeding Operation |
| UV | Ultra Violet |
| DNA | Deoxyribonucleic Acid |
| QMRA | Quantitative Microbial Risk Assessment |

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# CHAPTER I

# INTRODUCTION

## 1.1. Background

The human population is continually rising, reaching over 7.3 billion in 2015 according to the United Nations (2015). As the human population rises, consumption of animals and animal products also rises. The result of increasing human population and consumption is inevitably larger amounts of human and animal waste. Presently, most human waste in urban areas is collected and treated by wastewater treatment plants (WWTPs) in developed countries, and eventually released. A portion of animal waste, usually bovine waste, is sometimes collected by feedlots and dairy facilities for waste processing and possibly sold as manure for land application as a fertilizer. It must be noted that neither of these systems are closed systems, and bacteria and nutrients are often times released to the surrounding environments (nearby fields, crops, water bodies, aquifers, etc.) through runoff or an identified source. Water bodies are of particular interest as they can transport bacteria and nutrients to downstream aquatic environments and pollute otherwise clean waters.

The U.S. Clean Water Act of 1972 sought to identify polluted water bodies based on specific biological and chemical parameters, and outlined water quality standards. The 303(d) List of Impaired Waters was created to identify polluted waters by stating their specific impairment. As of 2014, there are 243 segments of water bodies in Texas listed on the 303(d) List due to bacterial contamination (TCEQ, 2014). Some water segments located downstream from Confined Animal Feeding Operations (CAFOs) and Wastewater

Treatment Plants (WWTPs) are listed on the 303(d) List due to bacterial contamination, specifically *Escherichia coli* (*E. coli*). The Environmental Protection Agency (EPA) has set a standard of 0 colony forming units (CFU)/100mL of *E. coli* for drinking water and 126 CFU/100mL for recreational water. Recreational water bodies that do not meet this requirement are placed on the 303(d) List of Impaired Waters.

### 1.2. *E. coli* phylotyping

*E. coli* is a common bacterial component of human and animal waste, and is currently used as an indicator organism for other possible pathogenic bacteria, viruses, and protozoa present from fecal origin, partly due to its ease of culturing. *E. coli* was discovered in 1885 by Theodor Escherich and is a Gram-negative, rod-shaped, facultative anaerobe in the Enterobacteriaceae family. There are hundreds of strains within the *E. coli* species; most current classification methods separate pathogenic from non-pathogenic strains. Classification methods include serotyping, pathotyping, and more recently phylotyping. Serotyping is a common classification method that relies on major surface antigens O, H, and K. The O antigen describes part of the lipopolysaccharide layer, the H antigen describes any flagella, and the K antigen describes the capsule (Orskov et al. 1997). Used more recently, pathotype (virotype) classification focuses on disease causing virulence factors and *E. coli* can be grouped into seven pathotype categories: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC) - also known as shiga toxin producing *E. coli* (STEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), diffusively adherent *E. coli* (DAEC), and uropathogenic *E.*

*coli* (UPEC). There is also avian pathogenic *E. coli* (APEC). All pathotypes are considered harmful, and pathotype classification has been used to assist researchers in understanding *E. coli* infections. Although helpful to understand the mechanism behind *E. coli* adherence and infection, pathotyping lacks detailed information on non-pathogenic strains, such as host origin.

Phylotyping was first used by Herzer et al. (1990). They took 72 *E. coli* isolates and applied multilocus enzyme electrophoresis (MLEE) to analyze mobility of 20 different enzymes. By using the neighbor joining method they created a phylogenetic distribution of the 72 strains and grouped them into four phylogroups: A, B1, B2, and D (Herzer et al, 1990). Ribotyping was also used to subtype *E. coli* strains into phylogroups (Blingen et al., 1994; Desjardins et al., 1995); however, both ribotyping and MLEE were considered time consuming and both required the use of an *E. coli* reference library for analysis (Clermont et al., 2000). Clermont et al. (2000) developed a faster method to determine phylogroups placement by analyzing the DNA of *E. coli* strains within and between phylogroups. They discovered two genes (*chuA* – outer membrane hemin receptor; *yjaA* – uncharacterized protein) and one DNA fragment (TspE4.C2 – part of putative lipase esterase gene) that acted as screens for phylogroup analysis. They validated their discovery by comparing its accuracy to the original 72 *E. coli* isolates tested by Herzer et al. (1990), and an additional 158 *E. coli* isolates previously phylogrouped using MLEE or ribotyping (Clermont et al., 2000). Clermont et al. (2000) created a decision tree to visualize how the presence or absence of each gene or DNA fragment would determine phylogroups classification. If the DNA of the *E. coli* strain contained

the gene *chuA,* it would either belong to phylogroup B2 or D. If the same *E. coli* strain DNA contained the gene *yjaA*, it would be classified as B2, if it didn't it would be classified as D. Alternatively, if the DNA of particular *E. coli* strain did not contain the gene *chuA,* it would belong to either B1 or A. If that same strain contained the DNA fragment TspE4.C2, it would be classified as B1, if it didn't it would be classified as A.

This new method, termed triplex polymerase chain reaction (PCR), eliminated the need for a reference library and thereby reduced analysis time significantly. Clermont et al. (2013) refined his method by developing more precise primers for *chuA, yjaA* and TspE4.C2, and adding primers for new phylogroups C and E (*arpA* – ankyrin-like regulatory protein; *trpA* – typtophan synthase) (Table 1). This new quadruplex PCR method allowed for more discriminatory power, grouping *E. coli* strains into seven phylogroups (A, B1, B2, C, D, E, F) and various clades (I-V), instead of only four phylogroups (Table 1) (Clermont et al., 2013). The method was validated using the 230 strains from Clermont et al. (2000), they found that quadruplex PCR identified the correct phylogroups 95% of the time compared to MLEE and MLST classification (Clermont et al., 2013).

The advantages of using phylotyping include the ability to quickly group *E. coli* strains using quadruplex PCR, the insight of pathogenicity, origin material, and preferred environmental habitat (Alm, 2011; Tenaillon, 2010). Researchers found that phylogroups B2 and D contained strains responsible for infection (Picard et al., 1999; Johnson & Stell, 2000); therefore, phylogroups B2 and D are considered potentially pathogenic. Humans are more likely to be hosts to phylogroups A and B2, and domesticated animals

predominantly host phylogroups A and B1 (Zhang et al., 2002; Gordon & Cowling, 2003; Escobar-Paramo et al., 2006; Skurnik et al., 2006; Baldy-Chudzik et al., 2008; Tenaillon et al., 2010). However, geographical location can also have an effect on dominant phylogroups that humans contain: in the United States B2 is the most dominant phylogroup (48%), in Pakistan it's A (47%), in Mali it's B1 (58%), and in France it's almost equal among A (25.5%), B1 (21%), D (24%), and B2 (29.5%) (Zhang et al., 2002; Nowrouzian et al., 2009; Duriez et al., 2001; Obata-Yasuoka et al., 2002).

There are, however, disadvantages to using phylotyping as a classification method. Using phylogroups that depend on four genes and a DNA fragment lacks specificity when attempting to identify virulence factors (and therefore possible pathogenic strains). *E. coli* as a species is known to exhibit genomic plasticity, and virulence genes could easily be transferred to an *E. coli* strain previously thought to be harmless. Additionally, one of the most widely known infectious *E. coli* strains, serotype O157:H7 is in phylogroup E instead of one of the potentially pathogenic phylogroups B2 or D. Nevertheless, we chose to use the Clermont quadruplex PCR method, mostly because this method facilitated the classification of more than 500 *E. coli* strains instead of limiting classification to 100-200 *E. coli* strains and searching for numerous virulence genes. This method allows us to analyze potentially pathogenic and non-pathogenic phylogroups alike.

**Table 1.** Phylotype assignment (adapted from Clermont et al., 2013).

| Phylotype | *arpA* | *chuA* | *yjaA* | TspE4.C2 | *trpA* (C) | *arpA* (E) |
|---|---|---|---|---|---|---|
| A | + | - | - | - | | |
| B1 | + | - | - | + | | |
| B2 | - | + | + | +/- | | |
| B2 | - | + | - | + | | |
| F | - | + | - | - | | |
| C | + | - | + | - | + | |
| A | + | - | + | - | - | |
| E | + | + | - | - | | + |
| D | + | + | - | +/- | | - |
| E | + | + | + | - | | + |
| Clade I | + | + | + | - | | - |
| Clade I or II | - | - | + | - | | |
| Clade III, IV, or V | - | + (476) | - | - | | |

Previous studies have shown strains belonging to groups B2 and D are more likely to cause extra-intestinal infections (Clermont et al., 2011); both are classified as potentially pathogenic phylotypes. The remaining phylotypes are considered non-pathogenic (A, B1, C, D, E, F). Phylotyping *E. coli* colonies from a variety of sources could shed light on potential pathogenicity present in fecal, soil, and water samples. This research focuses on dairy and human waste management systems.

### 1.3. *E. coli* in dairy manure management systems

Dairy cattle can produce up to 37 kg of waste per day (NCRS, 1992). As there are over 9 million dairy cattle in the US (Blayney, 2002), this can amount to over 333 million kg of manure every day that needs to be managed. Larger dairy farms require a management system to recycle and/or dispose of materials. Nutrient levels and fecal bacterial counts

must be monitored, as they could impact herd health and downstream applications (due to off-farm runoff movement). Cattle manure is composed not only of bovine feces, but also nasal, blood, and skin excretions that all collect on the stall floor. If not handled correctly, bacteria and other microbes in dairy manure can leave the confines of the farm through water runoff, leakage, or land applied manure and contaminate water and food supplies (Pell, 1997). Cattle manure has been implicated as a major source of fecal contamination in non-point source agricultural runoff in watersheds, Hancock et al. (1997) found that 63% of feed lots tested positive for *E. coli* O157:H7. In animal waste, pathogenic *E. coli* can survive from 50 to over 300 days, depending on environmental factors such as temperature, aeration and nutrient availability (Rogers, 2005). McGarvey et al. (2004) studied a waste system in California consisting of initial manure, separator pit and lagoon. They sampled each step for aerobic, anaerobic, and coliform bacteria and found that plate counts for all three decreased from manure to separator pit, and separator pit to lagoon water (McGarvey et al., 2004). However, they did not isolate any *E. coli* from any sampling location or date. Additionally, they sampled only one farm. Kearney et al. (1993) used cattle slurry to study the effect of storage on bacterial concentration and found that mesophilic anaerobic digestion caused a rapid decline in concentrations of *E. coli, Salmonella typhimurium, Yersinia enterocolitica,* and *Listeria monocytogenes*. However, these experiments were performed in laboratory conditions and bacterial strains were inoculated into slurry. Furthermore, previous studies on *E. coli* in dairy manure have largely focused on overall *E. coli* abundance and have not examined the composition and diversity of *E. coli* in dairy manure systems (Nicholson et al., 2005; Hancock et al., 1997;

Semenov et al., 2009; Islam et al., 2005; Shepherd et al., 2007). In order to prevent *E. coli* contamination from cattle waste, dairy farm operators must carefully select a manure management system that effectively reduces *E. coli* concentrations. Even more specific, the manure management system should aim to reduce potentially pathogenic *E. coli* that could be released in runoff.

### 1.4. *E. coli* in wastewater treatment plant effluent

*E. coli* is also used as a water quality standard for treated effluent released from WWTPs. In the United States, humans generate around 380 L of wastewater per person per day (EPA, 2004). WWTPs treat 150 billion L of wastewater per day, and treated effluent is released to downstream water bodies (Drinan & Spellman, 2013; EPA, 1997). WWTPs in the United States generally employ preliminary, primary, and secondary treatment, followed by disinfection before releasing effluent. The goal of preliminary treatment is to remove large solids and material by using coarse screening and grit removal. Primary treatment aims to remove organic and inorganic solids by sedimentation and skimming. The goal of secondary treatment is to remove biological matter, degradable carbon, by means of activated sludge (sludge with incorporation of bacteria, protozoa, and macro invertebrates). Finally, the goal of disinfection is to eliminate pathogenic microorganisms, such as pathogenic strains of *E. coli*, by use of chlorination or UV irradiation before releasing effluent.

Chlorination disinfection operates by oxidizing cell materials such as nucleic acids, and breaks down cell walls (EPA, 2004). Chlorine gas is injected into effluent and it reacts to produce HOCl and $OCl^-$, as seen in the equations below:

Eq 1: $Cl_2 + H_2O \leftrightarrow HOCl + HCl$

Eq 2: $HOCl \leftrightarrow H^+ + OCl^-$

The $OCl^-$ and HOCl are free chlorines and highly reactive with bacterial cell membranes. Direct hypochlorite ($OCl^-$) can also be applied instead of chlorine gas. A contact time of 15 to 60 minutes is required for significant reduction in bacterial concentration, dependent upon chlorine dosage and wastewater characteristics. Total Suspended Solids (TSS), pH, ammonia concentration, Biological Oxygen Demand (BOD), and nitrite concentration can have an effect on the performance of chlorination on lowering bacterial concentration. Additionally, spores and protozoan cysts are resistant to normal chlorination doses, often requiring longer contact times and higher levels of chlorine to be effective (EPA, 1999). Dechlorination is often employed after chlorination to remove harmful unwanted byproducts of chlorination. Dechlorination involves using sulfur dioxide, sodium bisulfite or sodium metabisulfite to remove free and combined chlorine residuals (EPA, 1999).

UV disinfection operates by a different (physical) mechanism and works by emitting rays at 254 nm that disrupt DNA within cells by creating pyrimidine dimers, thereby preventing any future replication (Figure 1) (Quek & Hu, 2008).

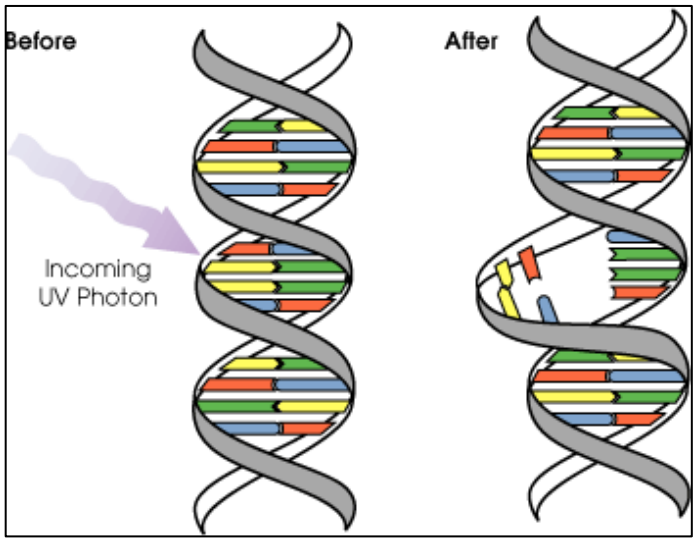


**Figure 1.** UV irradiation effect on microbial DNA
(Source: http://earthobservatory.nasa.gov/Features/UVB/).

Low pressure lamps emit monochromatic rays at 254 nm, and medium pressure lamps emit polychromatic rays between 200 – 300 nm to disrupt cellular DNA. However, humic substances, pH and TSS can affect UV ray penetration through water (EPA, 1999). Additionally, some bacteria are capable of activating repair mechanisms that can return damaged DNA to its original state (Quek & Hu, 2008). Nevertheless, the lack of chlorine residuals released in effluent has been an attractive advantage and many WWTPs in the United States have adopted this new disinfection method.

Despite both chlorination and UV irradiation's ability to significantly reduce bacterial concentrations, *E. coli* has been detected post-disinfection in both (Anastasi et al., 2013; Elmund et al., 1999; Bitton, 1994). Genetic variability in *E. coli* may lead to variability in which phylotypes survive the disinfection process and to what degree. The

need to identify which *E. coli* phylogroups are dominating wastewater effluent post-disinfection is crucial, especially if treated effluent harbors viable *E. coli* cells. Comparing *E. coli* phylogroup distribution between disinfection methods of chlorination and UV-irradiation could identify which disinfection is more effective at reducing potentially pathogenic phylotypes. Only a few recent studies have phylogrouped *E. coli* from WWTP effluent (Frigon, 2013; Anastasi et al., 2010; Anastasi et al., 2013); however, they utilized the Clermont triplex PCR method (four phylogroups) instead of the Clermont quadruplex PCR method (nine phylogroups).

### 1.5. *E. coli* repair mechanisms after UV-irradiation in WWTP effluent

WWTPs in the U.S. primarily employ chlorination or UV irradiation for disinfection; presently UV irradiation is gaining popularity due to its simplicity in operating and lack of chlorine residuals affecting downstream environments. However, UV irradiation does have a major disadvantage, after UV irradiation some bacterial cells are capable of repairing themselves through photoreactivation and nucleotide excision repair (Guo et al., 2011; Guo et al., 2012; Giannakis, 2014). UV irradiation disinfection operates by using UV-C (shorter wavelength) light at 254 nm, as it creates lesions in microbial DNA, termed pyrimidine dimers (Harm, 1980; Friedberg et al., 1995). The pyrimidine dimers prevent future translation and replication and thus inactivates the microbe.

However, some microorganisms with UV induced pyrimidine dimers are able to repair the damage using light or dark repair mechanisms. Light repair, also known as photoreactivation, is light-dependent and requires the activation of the enzyme photolyase.

Photolyase locates the pyrimidine dimer, binds to the DNA, then with natural light at 345-400 nm wavelength photolyase changes its configuration and breaks apart the pyrimidine dimer (Walker, 1984; Oguma et al., 2001). The microorganism is returned to its original state and can replicate. Dark repair after UV damage, also known as nucleotide excision repair, requires the coordination of a dozen different enzymes. These enzymes activated by the microbe create a complex that excises a large section of DNA surrounding the pyrimidine dimer (see Figure 2). Afterwards, DNA on the excised region is replaced by complementary bases from the unexcised region opposite by DNA polymerase and DNA ligase.

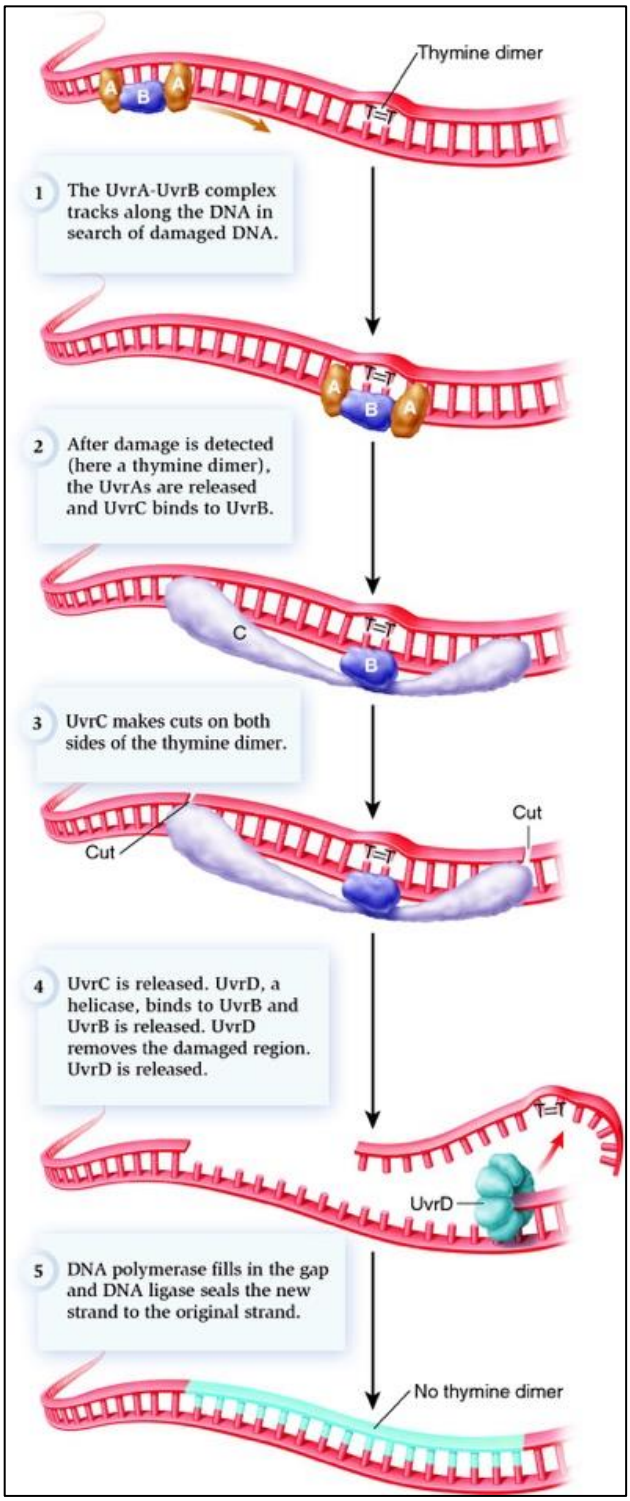


**Figure 2.** Nucleotide excision repair on microbial DNA after UV irradiation (Source: http://biology-forums.com/index.php?action=gallery;sa=view;id=468)

Photoreactivation and dark repair pose a problem for the efficacy of using UV-irradiation for disinfection at WWTPs (Sanz et al., 2007). Treated WWTP effluent is often used for agricultural purposes, landscape irrigation and golf course irrigation (EPA, 2016). Treated effluent from WWTPs utilizing UV irradiation is released downstream and is subject to environmental conditions that may be conducive to photoreactivation and/or dark repair. Many studies have found *E. coli* capable of both repair mechanisms (Quek & Hu, 2008; Whitby & Palmateer, 1993; Zimmer & Slawson, 2002; Harris et al., 1987). Quek and Hu (2008) analyzed *E. coli* photoreactivation and dark repair over a 4-hour period after UV-irradiation and found that *E. coli* photoreactivation ranged from 10 to 85% and *E. coli* dark repair ranged from 13 to 28% (2008). They also compared non-pathogenic *E. coli* to *E. coli* O157:H7 and discovered that the non-pathogenic *E. coli* exhibited higher levels of repair compared to *E. coli* O157:H7 (Quek & Hu, 2008). This leads to the possibility that potentially pathogenic phylotypes may have different repair rates than non-pathogenic phylotypes. Photoreactivation and dark repair after UV-irradiation have been documented with other organisms such as *Giardia muris* (Belsovic et al., 2001), *Strepococcus faecalis* (Harris et al., 1987), and fecal coliforms (Salceda et al., 2007). The majority of recent studies focused on the relationship between UV dose and *E. coli* repair rates, and few have analyzed samples directly from WWTPs. No studies to date have analyzed *E. coli* phylotypes from UV treated WWTP in subsequent dark and light conditions, or the phylotype distribution of surviving and repairing *E. coli* after UV irradiation. It is vital to understand the potential survival of possible pathogenic *E. coli* released to downstream environments.

### 1.6. Quantitative Microbial Risk Assessment (QMRA)

In addition to phylotyping *E. coli*, a Quantitative Microbial Risk Assessment (QMRA) would be helpful to analyze the potential risk associated with swimming in recreational waters located downstream from a WWTP. A QMRA is a way to model microbial contaminant behavior, it involves the identification of the hazard (in this case, *E. coli*), dose-response relationship (how many cells are needed to infect individual), assessment of exposure (when does a person come into contact with microbe and how much is ingested – i.e. air, water, solid), and the characterization of the hazard and the risk (the use of a mathematical model to predict risk) (Haas, 1999). *E. coli* is a well-documented microorganism in recreational waters, and can be hazardous by causing gastrointestinal illness, depending on which strain is ingested. Previous researchers have modeled *E. coli* in sea water near beaches (Schoen & Ashbolt, 2010; Gerba et al., 2000), compared *E. coli* in animal and human fecal contamination in water (Soller et al., 2010), and through the wastewater treatment process in Sweden (Westrell et al., 2004). It would be beneficial to understand the risk associated with using water bodies downstream from WWTPs for recreational purposes, especially if the segment is on the 303 (d) List of Impaired Waters.

### 1.7. Objectives

The ultimate goal of this research was to analyze the phylotype distribution of *E. coli* from different waste management systems and determine any trends related to potentially pathogenic *E. coli* phylotypes that could affect downstream environments. The two specific water sections examined in this study were in Texas, in Leon and Brazos counties.

Both are on the 303(d) List due to *E. coli* contamination, and both are located downstream of a CAFO or WWTP. Specifically, the objectives were to:

**1)** Analyze *E. coli* phylotype distribution across dairy manure management practices. Four dairy farms in central Texas located upstream from water sections on the 303(d) List due to bacterial (*E. coli*) contamination were selected. Previous analysis of *E. coli* concentration and isolate collection was completed by Dr. Emily Martin between 2009 and 2010.

**2)** Compare the difference in *E. coli* phylotype diversity in effluent using UV irradiation versus chlorination disinfection methods. Additionally, conduct a QMRA to assess risk to humans using downstream waters for recreational purposes.

**3)** Enumerate *E. coli* concentration and determine phylotype composition after UV disinfection, in light and dark treatments, and find corresponding *E. coli* phylotype photoreactivation and nucleotide excision repair rates over a 48-hour period.

# CHAPTER II

# EFFECTS OF DAIRY MANURE MANAGEMENT PRACTICES ON *E. COLI* CONCENTRATION AND DIVERSITY

## 2.1. Introduction

Dairy cattle can produce up to 37 kg of waste per day (NCRS, 1992). As there are over 9 million dairy cattle in the US (Blayney, 2002), this can amount to over 333 million kg of manure every day that needs to be managed. Larger dairy farms require a management system to recycle and/or dispose of materials. Nutrient levels and fecal bacterial counts must be monitored, as they could impact herd health and downstream applications (due to off-farm runoff movement). Cattle manure is composed not only of bovine feces, but also nasal, blood, urine, and skin excretions that all collect on the stall floor. If not handled correctly, bacteria and other microbes in dairy manure can leave the confines of the farm through water runoff, leakage, or land applied manure and contaminate water and food supplies (Pell, 1997). Land application of manure or using dairy wastewater for crop irrigation has the potential to contaminate crops used for human consumption with potentially harmful microorganisms including pathogenic strains of *E. coli* such as *E. coli* O157:H7 (Nicholson et al., 2005).

Cattle manure has been implicated as a major source of fecal contamination in non-point source agricultural runoff in watersheds. Manure can be carriers of a variety of zoonotic pathogens, including bacteria, viruses, and protozoa. In fact, concentrated animal feeding operations (CAFOs) commonly harbor pathogenic strains of *E. coli*. Hancock et

al. (1997) found that 63% of feed lots tested positive for *E. coli* O157:H7. In animal waste, pathogenic *E. coli* can survive from 50 to over 300 days, depending on environmental factors such as temperature, aeration and nutrient availability (Rogers and Haines, 2005). Khaleel (1980) noted that higher concentrations of animal facilities located near water sources could lead to surface water contamination. Johnson (2003) discovered that sampling sites located downstream of agricultural activities tested positive for *E. coli* O157:H7. Bovine manure and manure slurry may pose a health risk when applied as a nutrient source for crops (Himathongkham et al., 1999). Additionally, *E. coli* O157:H7 can survive up to 8 weeks in moist soil at 25°C (Mubiru et al., 2000). Guan & Holley (2003) found that *E. coli* O157:H7 was the most persistent bacterium studied in cow manure, as compared to *Salmonella, Campylobacter, Yersinia, Cryptosporidium,* and *Giardia,* at any temperature studied and in either solid or slurry form.

*E. coli* is of particular importance due to its use as a water quality indicator by regulatory agencies. *E. coli* is naturally present in the intestines of warm-blooded animals and most strains are beneficial to gut functionality. However, some strains, such as the aforementioned *E. coli* O157:H7, are pathogenic and present manure managers with pressing issues on how to reduce abundance and pathogenicity. Previous studies on *E. coli* in dairy manure have largely focused on overall *E. coli* abundance and have not examined the composition and diversity of *E. coli* in dairy manure systems (Nicholson et al., 2005; Hancock et al., 1997; Semenov et al., 2009; Islam et al., 2005; Shepherd et al., 2007). One study conducted by Lu et al. (2005) showed greater *E. coli* diversity in a mixing tank, as a second step in the manure management practice, than when initially

collected from the barn floor. Substructure within the *E.coli* species allows for phylogroup classification, which gives insight into pathogenicity, origin material, and preferred environmental habitat (Alm & Walk, 2011). *E. coli* phylotyping began in 2000 with triplex PCR which screened for two genes, and one DNA fragment: *chuA, yjaA*, and TspE4.C2, respectively. This process resulted in phylogroup classification of A, B1, B2, and D. Since then, a more advanced method evolved to distinguish between seven phylogroups (A, B1, B2, C, D, E, and F) and various clades using quadruplex PCR (Tenaillon et al., 2010; Clermont et al., 2013). Quadruplex PCR involves screening for the original genes and DNA fragment with more specialized primers, along with an additional gene (*arpA*), and primers for new groups C and E (Clermont et al., 2013).

Previous studies have shown that groups B2 and D can cause extraintestinal infections; both are classified as pathogenic phylotypes (Clermont et al., 2011). Groups B1 and A are most dominant in mammals, B1 originates primarily from feces (Clermont et al., 2011; Dixit et al., 2004). Phylogroup B2 has been documented in dairy waste and may be responsible for downstream contamination and infection (Tenaillon et al., 2010).

Previous studies indicated that as animal waste moves through a management system, such as from scrape pile to separator pit to lagoon, *E. coli* levels were significantly reduced (along with heterotrophic plate counts) (McGarvey et al., 2004; Peu et al., 2006). Although these studies indicate that management systems can reduce levels of *E. coli* in dairy waste or lagoons, they provide limited information on which *E. coli* populations persist in dairy waste.

The objective of this research was to determine how management practices impacted *E. coli* concentration and phylotype distribution throughout four different dairy manure management systems in central Texas. The results could provide valuable information on how these systems not only reduce *E. coli* concentration but also how they impact the diversity of *E. coli* populations, including the presence of potentially pathogenic *E. coli* strains, and provide vital information for guiding quantitative microbial risk assessment-based approaches for evaluating the efficacy of waste management strategies (Soller, 2006).

## 2.2. Materials and methods

### 2.2.1. Sample collection for *E. coli* enumeration

In 1996, the Leon River in Texas was placed on the § 303(d) List of impaired waters due to bacterial contamination, with fecal contamination from upstream dairy farms being possible source contributors (TCEQ, 2014). Sampling of four dairy farms in the Leon watershed was conducted in 2009 by Dr. Emily Martin. Manure management practice varied at each dairy farm, including a combination of lagoons, separators, drying beds, and piles (Figure 3). Dairy 1 scraped manure from pens into a pile (prior to land application), and a slurry into a lagoon. Dairy 2 operated a flushing system that used water to wash manure into a settling basin to remove some solids, then liquids from the settling basin were pumped into a lagoon. Dairy 3 scraped some manure into a pile; the remaining manure went into a separator that removed bedding material, then the liquids were sent to

a lagoon. Dairy 4 vacuumed raw manure into a slurry pond which allowed gravity separation of solids from liquids, slurry solids were spread on a drying bed, liquids were sent to a lagoon.

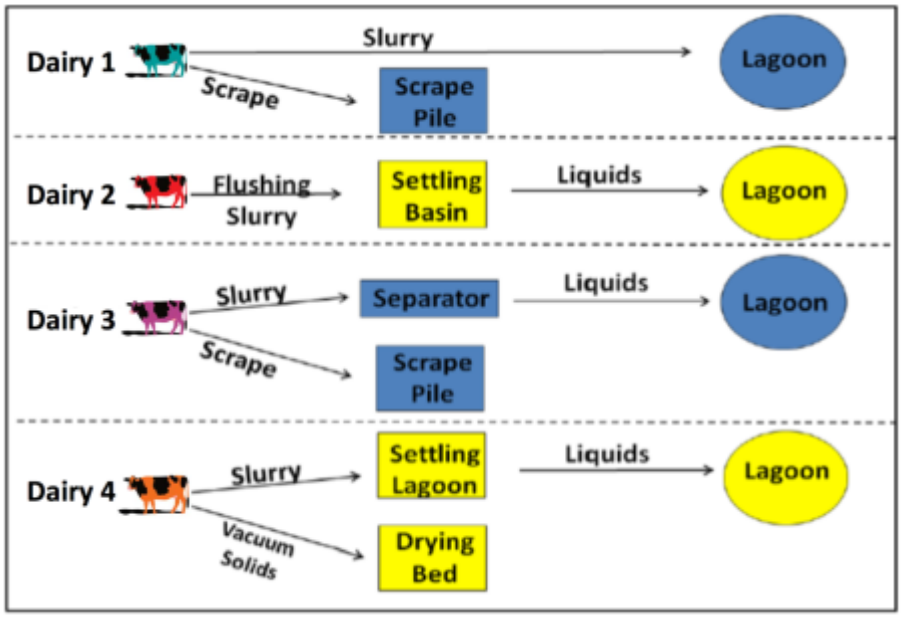


**Figure 3.** Diagram of manure management practices employed at four dairies in this study.

Samples were taken the first week of January, April, July, and September of 2009 from each stage by Dr. Emily Martin. Five to ten representative samples in each treatment area were collected and mixed to form a composite sample. Whirl-Pak® bags or autoclaved Nalgene® bottles were used to collect samples that were transported on ice back to the lab and processed for *E. coli* enumeration within 6 hrs, according to EPA Method 1603 (modified membrane-Thermotolerant *Escherichia coli,* also known as mTEC). Filtered

samples were incubated at 35°C ± 0.5°C for 2 hr, then incubated at 44.5°C ± 0.2°C for 22 hrs. After 24 hrs, mTEC plates were counted and red-magenta colonies were considered *E. coli* colonies. Up to five *E. coli* colonies were randomly selected and isolated from each mTEC plate and transferred onto EC-MUG medium (EMD, Gibbstown, NJ) as a secondary screen for β-glucuronidase enzyme activity. After 24 hrs incubation, colonies were isolated and stored in tryptic soy broth with 20% glycerol at -80°C for future polymerase chain reaction (PCR) analysis.

2.2.2. Phylotyping

After 24 hr growth on EC- MUG plates at 35°C ± 0.5°C for 2 hr, single colonies were isolated from each plate with a 1 µL loop and transferred into 100 µL molecular grade sterile water (cell suspension). Each PCR reaction included 12.5 µL of GoTaq G2 Green Master Mix, 1.5 µg/µL bovine serum albumin, 4.5 µL of molecular grade $H_2O$, 5 µL of DNA template (from cell suspension as described above), 1 µL of forward primer, and 1 µL reverse primer (at a concentration of 20 pmol/µL solution). PCR conditions consisted of initial denaturation for 10 minutes at 94°C; followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds; final elongation of 7 minutes at 72°C; and holding at 4°C until removed from the Eppendorf Mastercycler. Resulting PCR products were visualized using a 2% agarose gel prepared with 1X TBE buffer and 0.5 µg/mL ethidium bromide. DNA ladders (exACTGene Low Range Plus DNA Ladder) were used in the first and last well of each gel for base pair length comparison, along with positive and negative controls for each

gene segment targeted. Electrophoresis occurred at 120V for 25-35 minutes. Gels were imaged with a UltraLum Omega Molecular Imaging system.

Quadruplex phylotyping using the results from genes *arpA*, *chuA*, *yjaA*, and gene fragment TspE4.C2 were done using previous assignment as described by Clermont et al. in 2013. Primers included: *chuA* forward 5'-ATCTGGATGGTATTGTGGCCTGGT-3' and *chuA* reverse 5'-AGTTTCCGGACGTAAGTTCGGGTT-3', 288 bp product; *yjaA* forward 5'-ATGTCAGTTCTGTATATCCAAATTCGTCG-3', *yjaA* forward 5'-ATTAGTATTCGCCGCTCACG -3', 211 bp product; TspE4.C2 forward 5'-GGCACTGGAAAAAGGAATTGC-3', TspE4.C2 reverse 5'-TTACCTTCCCGCTCTCCAGG-3', 154 bp product; *arpA* forward 5'-AACGCTATTCGCCAGCTTGC-3', *arpA* reverse 5'-TCTCCCCATACCGTACGCTA-3', 400 bp prpduct; *arpA* (E) forward 5'-GATTCCATCTTGTCAAAATATGCC-3', *arpA* (E) reverse 5'-GAAAAGAAAAAGAATTCCCAAGAG-3', 301 bp product; *trp* (C) forward 5'-AGTTTTATGCCCAGTGCGAG-3', *trp* (C) reverse 5'-TCTGCGCCGGTCACGCCC-3', 219 bp product. A portion of the isolates required additional screening using C and E primers to distinguish between A or C, D or E, and E or clade I (see Table 1 in chapter I for phylotype assignment).

### 2.2.3 Statistical analyses

Dr. Emily Martin compared *E. coli* CFU/g or mL using log transformed concentration in an ANOVA, presented below for comparison to phylotype data. Proportions of

phylotypes were compared in Excel using a two-tailed t-test of all three or nine runs, with statistical significance identified with a p-value $\leq 0.05$.

## 2.3. Results and discussion

### 2.3.1. *E. coli* concentration in dairy waste streams

As previously reported by Dr. Emily Martin (2013), as the waste moved through the management system, *E. coli* concentrations decreased (Figure 4). *E. coli* numbers from drying bed to the settling lagoon drastically reduced in dairy 4, about a 3 log reduction in *E. coli* concentration. This may be attributed to the separation of the solid and liquid waste components and the high temperature of the drying bed desiccating the *E. coli*. *E. coli* concentration was also reduced in dairy 3 from scrape to lagoon, to a lesser extent than dairy 4. The manure management systems that did not separate solid from liquid waste, seen at dairies 1 and 2, did not significantly ($p > 0.05$, one-way ANOVA with log transformed values) reduce *E. coli* concentrations, although a general decreasing trend can be seen.

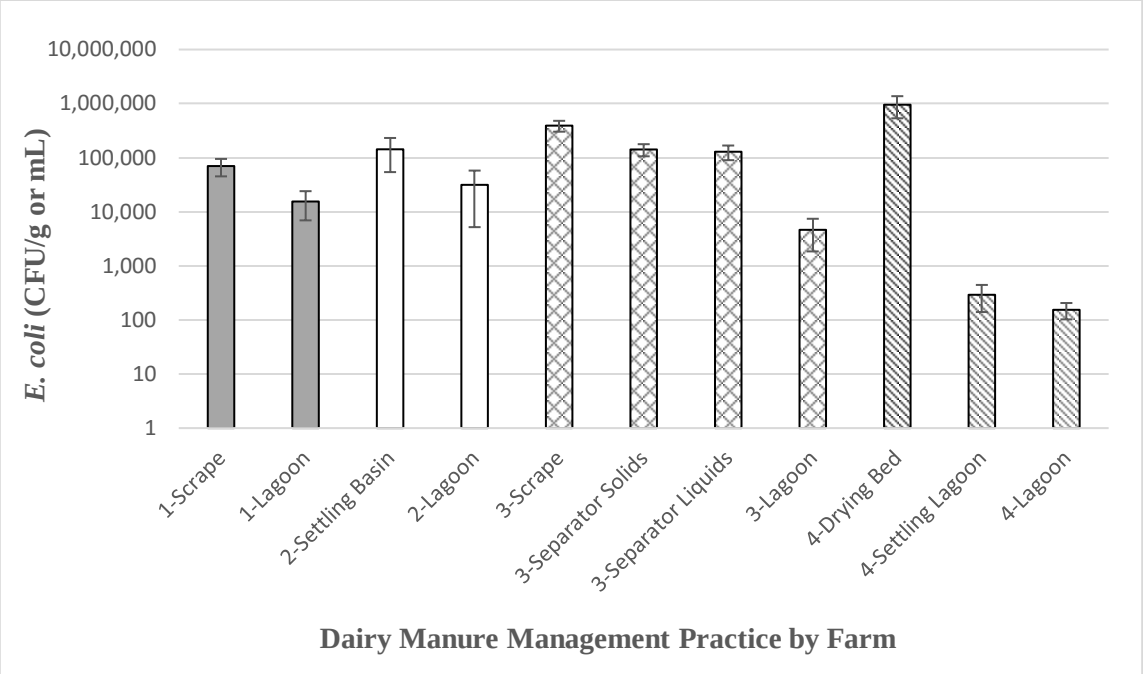


**Figure 4.** Average *E. coli* concentration (CFU/g or mL) for each dairy manure management over a one-year period (sampling January, April, July, September). Manure samples are shown as CFU/g, and lagoon and liquid samples are shown as CFU/m. Data obtained by Dr. Emily Martin (Martin, 2013).

However, due to a herd reduction in May 2009, fewer samples were collected from dairy 1. Of the dairies we studied, those with more advanced methods for separation of manure solids had the lowest concentrations of *E. coli* in the final treatment lagoons. The separation of solids from liquid waste facilitates the removal of *E. coli* cells with the solid portion. Stress is placed on the remaining *E. coli* cells as they move through three to four different environments, instead of one to two as seen in the non-tiered manure management systems. *E. coli* moving through tiered manure management systems must survive multiple stress-inducing environments including a combination of scraping,

vacuuming, washing, and drying, which can cause mechanical sheering, lysing, and desiccation of cells. McGarvey et al. (2004) observed a similar decreasing trend with aerobic and anaerobic bacteria as they traveled through a waste management system from manure to separator pit to lagoon. Environmental conditions at each stage were uniquely different and coliform plate counts were lower in the lagoon system than the separator pit, which agrees with our results.

No consistent seasonal trends in *E. coli* levels were observed during the study (data not shown). Although Shere et al. (1998) conducted a study on cattle waste in 1998 and found higher levels of *E. coli* O157:H7 in spring and late summer, we did not find similar results, with either overall or potentially pathogenic *E. coli* levels. The length of time waste spent at each stage of the management process could have had an effect on *E. coli* survival, as over 99% of *E. coli* was reduced in seven days of bovine waste in a previous study, however this variable was not measured in our study (Shere et al., 1998).

### 2.3.2 *E. coli* phylotypes across dairies

The most common phylotype across all dairies and time periods was B1 (Table 2), which was seen at each dairy and with each treatment. Additionally, B1 was seen with almost equal frequency throughout the manure management system (68% average), followed by C (13.9%), A (13.6%), D (8.9%), E (8.4%), unknown (4.6%), B2 (1.3%), and finally F (0.6%). These results agree with previous research by Badouei et al. (2015) who isolated *E. coli* from cow waste and found B1 to be the most dominant phylotype. Another study conducted by Cook et al. (2011) sampled and phylotyped *E. coli* from dairy cattle feces

and also found the majority of phylotypes to be B1. Escobar-Paramo (2006) found similar results when comparing 1898 isolates from 387 mammals, phylotypes B1 and A were most prevalent, followed by D and B2 when using the triplex Clermont method. Phylotypes B1 and A were also the most common from our samples, at each dairy and manure management stage. Baldy-Chudzik (2008) conducted a study identifying *E. coli* phylotypes in herbivore feces and most of the isolates were grouped as B1. One reason for high B1 proportions could be their attachment efficiency. Attachment efficiency is a bacterium's ability to attach to a surface, and is usually measured by flow through a column of quartz sand, as done by Cook et al. (2011). Phylotype B1 isolates in dairy waste, as compared to swine waste, poultry waste, and stream water, ranked mid to top in attachment efficiency (Tenaillon et al., 2010). Cook et al. (2011) noted that the highest ranked attachment efficiency isolates possessed all or a majority of the targeted genes for adherence. This could explain why the majority of *E. coli* found persisting in the environment are B1 (Cook et al., 2011; Walk et al., 2007).

The next most dominant phylotype we saw in our study was A. One possible explanation is that A and B1 are sister groups (Lecointre et al., 1998), and may share more similar genetic structure than any other phylotype. Phylotype A was present at every dairy and every manure management step, except for in dairy 1 – scrape treatment. Phylotype A prevalence has been previously documented by Alizade et al. (2014) in dairy and calf feces, with 40% of *E. coli* phylotypes as A.

**Table 2.** Phylotypes from each dairy treatment and dairy location represented as a percentage of total isolates collected at that specific location (each row equals 100%).

| | % Phylotype | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Location** | **A** | **B1** | **B2** | **C** | **D** | **E** | **F** | **Unknown** |
| Dairy 1-Scrape | - | 90 | - | 10 | - | - | - | - |
| Dairy 1-Lagoon | 27 | 67 | - | 6 | - | - | - | - |
| Dairy 2-Settling Basin | 5 | 80 | - | - | - | 15 | - | - |
| Dairy 2-Lagoon | 5 | 53 | - | 21 | 5 | 5 | 5 | 5 |
| Dairy 3-Scrape | 10 | 60 | 5 | 10 | - | 5 | - | 10 |
| Dairy 3-Separator Solids | 5 | 68 | - | 5 | 5 | 16 | - | - |
| Dairy 3-Separator Liquids | 15 | 75 | 5 | 5 | - | - | - | - |
| Dairy 3-Lagoon | 10 | 65 | - | 10 | - | 10 | - | 5 |
| Dairy 4-Drying Bed | 5 | 70 | - | 15 | - | 5 | - | 5 |
| Dairy 4-Settling Lagoon | 21 | 64 | - | 7 | - | - | - | 7 |
| Dairy 4-Lagoon | 5 | 55 | - | 20 | 5 | 10 | - | 5 |

Phylotype C was present throughout each dairy and management step, except for dairy 2 in the settling basin. An unknown phylogroup(s) appeared seven times through sampling, which would have required further multilocus sequence typing (MLST) involving the screening of 13 additional genes. The extensive additional testing required by MLST, and as the quadruplex PCR method correctly assigns 85-90% of strains as compared to MLST (Clermont et al., 2013), no additional testing was conducted. Almost 86% of these unknown phylotypes occurred in dairies 3 and 4, equally.

Very few of the potentially pathogenic *E. coli* phylotypes (B2 and D) were found in the study. Dairies 2 and 4 had low proportions (5%) of phylotype (D) in their lagoon system. Dairy 3 had the most occurrences of potentially pathogenic phylotypes (B2 & D),

including the only two occurrences of B2 in the entire sample set. Dairy 1 had no pathogenic phylotypes at any waste management step or sampling date, however, the low number of sampling dates, due to a herd reduction in May 2009, and reduced number of isolates from the samples could account for this trend. Gordon & Cowling (2003) sampled mammalian herbivores in Australia and found a higher concentration of B2 phylotypes, followed by B1, D and A. Although our results are not similar, this could be due to geographical location and diet. Furthermore, domestication of cattle may play a role in which phylotypes appear in dairy manure. Escobar-Paramo (2006) sampled feces from wild and farm animals and found that B2 strains are seen more in wild animals (30%) as compared to farm animals (14%), and A strains are seen more in farm animals (27%) as compared to wild animals (14%). One possible explanation for the more widespread B2 phylotypes seen in wild animals is their potential exposure risk. They have a much higher chance of interacting with other animals or animal waste that possess the B2 phylotype than farm animals that primarily interact with similar farm animals in a confined environment. Escobar-Páramo (2006) found that wild animals and humans shed more potentially pathogenic *E. coli* phylotypes than domesticated animals do, which may partly explain the low counts of potentially pathogenic *E. coli* across all dairies and manure management treatments.

Kim et al. (2009) found that indigenous microflora were largely responsible for the suppression of pathogenic *E. coli*. It is possible that pathogenic *E. coli* were suppressed as they moved through the manure management system, if fresh manure was mixed in with an established microbial community at each stage. The survival of

pathogenic *E. coli* could possibly depend on existing microflora, which was not analyzed in this research study. Maule et al. (2000) found that *E. coli* survival is significantly reduced in slurry as compared to manure, possibly due to protozoal grazing. Ibekwe et al. (2003) also found that healthy and diverse microbial communities were essential for efficient dairy wastewater treatment. Leaving a small portion of manure or slurry at each stage, with an established microbial community, could help to suppress pathogenic *E. coli* when mixed with incoming fresh manure.

Dairies 1 and 3 both employed scraping as a manure management step, and in both scrape treatments, B1 was the most dominant phylotype. The scrape treatment in dairy 3 exhibited more phylotype diversity, with 6 phylotypes present (A, B1, B2, C, E, Unknown) as compared to the two phylotypes observed in dairy 1 samples (B1, C). The lagoon treatment, however, showed the most diverse *E. coli* phylotypes as compared to samples collected from scrape pile, settling basins, and drying beds. Dairy 2 showed the highest phylotype diversity with 7 phylotypes present (A, B1, C, D, E, F, Unknown), and the lagoon at dairy 1 showed the least with only 3 phylotypes present (A, B1, C). This trend was observed in all studied dairies except for dairy 3. This could be due to decreasing abundance and diversity of surrounding aerobic and anaerobic bacteria, as found by McGarvey in 2004, as manure moves through the management system. They found that coliform plate counts decreased from $2.4 \times 10^6$ CFU/g to $1.1 \times 10^2$ CFU/mL as manure moved from its initial state to lagoon water (McGarvey et al., 2004). As abundance and diversity of surrounding bacteria decreases, surviving *E. coli* may have had more access

to nutrients and less predation, which may have led to higher levels of diversity in the lagoons.

## 2.4. Conclusion

This study is the first to use the Clermont quadruplex PCR method to determine phylotype distribution in dairy cattle waste as it travels through manure management systems. Further studies comparing *E. coli* phylotypes from manure management systems to those present in downstream water samples could help identify which phylotypes are surviving the transport process. Studying the potentially pathogenic *E. coli* phylotype concentration released from CAFOs is vital to understanding how to protect downstream aquatic environments.

All dairy manure management systems in this study were effective at reducing levels of *E. coli* in their waste process systems. Dairies that employed tiered manure management systems reduced *E. coli* concentrations the most. Phylotype B1 was the most common from each dairy at each treatment, followed by phylotypes C and A. Overall, potentially pathogenic phylotypes were not very common among *E. coli* colonies isolated from dairy manure samples (2.6% occurrence). A caveat to this research is the low number of isolates collected; future studies would benefit from sampling more often and collecting more isolates per sample, and sampling at various depths within each management system.

As of 2012, in Texas alone there were 257 segments of impaired water bodies due to bacterial contamination (TCEQ 2012). Proper management of source and non-point source bacterial pollution can have a significant effect on downstream water quality. Any

of the impaired water bodies located downstream from dairy farms have the potential to decrease bacterial contamination by implementing tiered manure management systems to reduce *E. coli* concentration.

# CHAPTER III

# *E. COLI* PHYLOTYPE DISTRIBUTION IN WASTEWATER TREATMENT PLANT EFFLUENT BEFORE AND AFTER CHLORINATION AND UV-IRRADIATION

## 3.1. Introduction

The average U.S. resident uses 375 liters of water per day (EPA, 2008). Wastewater treatment plants (WWTPs) provide an essential function in every community across the United States by collecting and treating used water (wastewater). There are over 16,000 publicly owned WWTPs that treat around 34 billion gallons of wastewater per day in the United States (Drinan & Spellman, 2013; USEPA, 2016).

Wastewater is treated in a series of processes: preliminary, primary, secondary, and disinfection. In preliminary treatment larger objects are removed from effluent by means of racks, bar screens and grit chambers. In primary treatment sedimentation is used to separate effluent from heavier particles which sink to the bottom. Light materials such as fats, grease, and plastic materials float to the top and are skimmed off for disposal. Secondary treatment involves aerating effluent and using activated sludge to consume organic materials in effluent, then using settling tanks to remove most of the heavier particles and activated sludge. Disinfection aims to remove harmful microorganisms by using chlorination or UV-irradiation. Treated wastewater (effluent) is released into water bodies (streams, rivers, lakes), that may be used for recreational purposes and/or recaptured downstream for drinking water use. Therefore, the quality of treated and released effluent is of primary concern.

Among bacteria found in wastewater, *Escherichia coli* (*E. coli*) is of primary interest. *E. coli* is a relatively easily cultured Gram-negative bacterium commonly found in the gut of warm-blooded animals. It is commonly used as an indicator organism for other possible pathogenic bacteria, viruses and protozoa present in fecal matter. The majority of *E. coli* strains are benign, however some types of *E. coli* are pathogenic, such as *E. coli* O157:H7, and can cause urinary tract infections, diarrhea, and even contribute to death (Berg, 2004).

Substructure within *E. coli* as a species gives insight into pathogenicity (Alm, 2011). Specifically, seven phylogroups of *E. coli* (A, B1, B2, C, D, E, F) and various clades (I-V) can be distinguished by using the Clermont quadruplex PCR method; the original PCR method was developed in 1990 by Herzer, and most recently refined in 2013 by Clermont et al. (Herzer, 1990; Clermont et al., 2013). Clades represent phylogenetically similar *E. coli* strains, previously undescribed, separate from any established phylogroup (Walk et al., 2009). Classification is assigned by the presence or absence of three genes and a DNA fragment (*arpA*, *chuA*, *yjaA*, and TspE4.C2, respectively), as seen in Table 1 (Chapter I). Phylogroups B2 and D are currently considered potentially pathogenic phylotypes (Clermont et al., 2011).

To eliminate or significantly reduce pathogenic microorganisms, WWTPs generally employ chlorination or UV irradiation for disinfection. Chlorination operates by oxidizing cell materials such as nucleic acids, and breaking down cell walls (EPA, 2004). UV disinfection works by emitting rays at 254 nm that disrupts DNA within cells by creating pyrimidine dimers, thereby preventing any future replication (Quek & Hu,

2008). Past research has documented the effectiveness of chlorination and UV irradiation on *E. coli* survival in WWTP effluent (Lindenauer & Darby, 1994; Rice et al., 1999; Childress et al., 2014; Kaur et al., 2013). Only a few recent studies have focused on the phylotype distribution of *E. coli* (Anastasi et al., 2010; Anastasi et al., 2013). It is possible that certain phylotypes may survive chlorination or UV-irradiation at higher proportions than other phylotypes. Understanding the *E. coli* phylotype distribution in WWTP effluent could give insight into impacts on downstream environments. Furthermore, discerning the survival of potentially pathogenic phylotypes from harmless phylotypes could lead to more precise Quantitative Microbial Risk Assessments (QMRAs) of using downstream waters for recreational purposes. A few recent studies have phylotyped WWTP effluent after disinfection (Anastasi et al., 2010; Anastasi et al., 2013); however, these few studies utilized the triplex-PCR method, which is less precise than the quadruplex PCR method. They also did not conduct a QMRA on WWTP effluent, to our knowledge this is the first study to do so using *E. coli* phylotype data.

Quantitative Microbial Risk Assessment (QMRA) is a helpful tool to analyze the potential risk associated with a particular microbe in a specific setting, and involves the identification of the hazard, assessment of exposure, and the characterization of the hazard and the risk. *E. coli* is a well-studied microorganism, and can cause gastrointestinal illness, depending on which strain is ingested. The EPA has set a standard of 126 CFU/100mL of *E. coli* in recreational waters. Conducting a QMRA on WWTP effluent gives a rough estimate of risk associated with using downstream waters for recreational purposes.

The objectives were to enumerate *E. coli* concentration and identify phylotype diversity in effluent from two WWTPs, that employ UV irradiation and chlorination disinfection to compare the disinfection methods, located upstream from two water segments placed on the 303(d) List due to bacterial (*E. coli*) contamination. Additionally, conduct a QMRA on effluent from both WWTPs to assess risk to humans using downstream waters for recreational purposes.

### 3.2. Materials and methods

For the UV-treatment, samples were collected before and after UV treatment at a municipal wastewater treatment plant (termed Site-UV) in central Texas on March 2, May 4, & May 18, 2015. Water characteristics were provided by WWTP personnel and are included in Table 3. Three grab samples of 800 mL were collected with Whirl-Pak bags (Nasco, Modesto, California) on each date, from before and after UV treatment. Samples were immediately taken back to the laboratory for processing following EPA Method 1603 (modified mTEC) (US EPA, 2002). Aliquots of 100 mL, 10 mL, 1 mL, and 0.1 mL were filtered for enumeration. Samples were incubated at 35°C ± 0.5°C for 2 hrs, then incubated at 44.5°C ± 0.2°C for 22 hrs. After 24 hours, mTEC plates were counted and red-magenta colonies were considered *E. coli* colonies. Up to ten *E. coli* colonies were randomly selected, using a random number generator, and isolated from each mTEC plate and transferred onto EC-MUG medium (EMD, Gibbstown, NJ) as a secondary screen for β-glucuronidase enzyme activity. After 24 hrs incubation colonies were isolated and stored in tryptic soy broth with 20% glycerol at -20°C for future PCR analysis.

For the chlorination disinfection treatment, samples were collected from a municipal wastewater treatment plant (Site-Chlor) located in central Texas on July 6, 13, and 21, 2015. Water characteristics are included in Table 4. On July 6, three samples from before chlorination treatment and after dechlorination were collected in Whirl-Pak® Bags and transported back to the lab immediately for processing using EPA Method 1603 (modified mTEC). On July 13 and 21, three samples were collected before chlorination treatment and six samples after dechlorination due to the small number of *E. coli* colonies isolated using only three 100 mL samples on the first sampling date. Enumeration, isolation and secondary confirmation were carried out as outlined above.

**Table 3.** Water characteristics for Site-UV.

| Sampling date | Flow (MGD) | TDS (mg/L) |
|---|---|---|
| March 2, 2015 | 6.0 | 570 |
| May 4, 2015 | 6.3 | 540 |
| May 18, 2015 | 6.4 | 440 |

**Table 4.** Water characteristics for Site-Chlor.

| Sampling date | Flow (MGD) | TSS (mg/L) | Chlorine (mg/L) |
|---|---|---|---|
| July 7, 2015 | 4.5 | 8 | 1.2-2.5 |
| July 14, 2015 | 4.6 | 4 | 1.2-2.5 |
| July 21, 2015 | 4.6 | 2 | 1.2-2.5 |

### 3.2.1. Phylotyping

*E. coli* colonies isolated using mTEC and MUG agar were stored in 0.1 mL aliquots of molecular grade sterile water (cell suspensions) at -20°C. PCR reactions included 12.5µL of GoTaq G2 Green Master Mix, 1.5 µg/ µL bovine serum albumin, 4.5 µL of molecular grade $H_2O$, 5 µL of DNA template (from cell suspensions), 1 µL of forward primer and 1 µL of reverse primer (primer concentration of 20 pmol/µL solution for each primer) (Table 4). PCR conditions (described by Clermont et al., 2013) included denaturation at 94°C for 10 min; followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec; final elongation at 72°C for 7 minutes; and holding at 4°C until removed from the Eppendorf Mastercycler (Hamburg, Germany). Gel electrophoresis occurred at 125V for 25-35 minutes, using a 2% agar gel prepared with 1 X TBE buffer and 0.5 µg/mL ethidium bromide. DNA ladders (exACTGene Low Range Plus DNA Ladder) were used in the first and last well of each gel for base pair length comparisons, along with positive and negative controls for each gene segment targeted, isolated from *E. coli* strains from a previous experiment. Gels were visualized using an UltraLum Imager (UltraLum, Carlsbad, California) and the presence or absence of targeted genes recorded. A second PCR reaction was conducted on colonies which had the classification of A or C, or E or D. C and E primers were used to finalize phylotype classification.

### 3.2.2. Quantitative Microbial Risk Assessment (QMRA)

In this study, exposure was calculated as *E. coli* CFU/100 mL in post-treated wastewater effluent from both WWTPs, multiplied by average water ingestion of 37 mL for children

in a single swimming/recreational event (Dufour, 2006). Characterization of the hazard relates the exposure level to the percentage of *E. coli* that can cause adverse health effects. Although determining exact pathogenicity is difficult, one definition relates *E. coli* pathogenicity to its ability to adhere and colonize human intestinal tissue (Duffy, 2006). However, adherence and colonization may not always result in illness. To assess phylotype pathogenicity, we chose B2 as the potential pathogenic phylotype and referenced a study conducted by Picard et al. (1999). *E. coli* strains were isolated, phylotyped, and then the mortality rates in mice was measured, resulting in 83.78% for B2 isolates (Picard et al., 1999). Finally, characterization of the risk shows the number of people that would exhibit symptoms of gastrointestinal illness after being exposed. We calculated exposure rate as follows: *E. coli* CFU/100 mL x % of B2 isolates x 83.78% pathogenicity rate x 37 mL ingestion = exposure dose. We selected the exponential model previously used for enterohemorrhagic *E. coli* due to its pathogenicity compared to general *E. coli* models (Cornick & Helgerson, 2004).

### 3.2.3 Statistical analyses

Means of treatments (*E. coli* CFU/100 mL, percentage of specific phylotypes, pathogenic phylotype percentage) were compared in Excel using a two-tailed t-test of all three or nine runs, with statistical significance identified with a p-value $\leq 0.05$.

## 3.3. Results and discussion

### 3.3.1 Total *E. coli* concentration

The total *E. coli* concentration was drastically reduced after chlorination and UV-irradiation (Figure 5). Effluent from Site-UV had a lower *E. coli* concentration before effluent entered into the disinfection step, averaging 5.1 x $10^3$ CFU/100 mL, as compared to chlorination (Site-Chlor). After UV irradiation, the average *E. coli* concentration dropped to 1.9 x $10^2$ CFU/100 mL. Effluent from Site-Chlor also had a higher concentration of *E. coli* before disinfection as compared to after, at 2.4 x $10^4$ CFU/100 mL and 2.2 x $10^2$ CFU/100 mL, respectively. The difference of incoming *E. coli* concentrations could be due to a variety of factors, including wastewater source (different populations served. Both disinfection methods significantly reduced *E. coli* concentrations. In 1999, Elmund et al. sampled effluent at two WWTPs in Colorado (one using chlorination and the other UV irradiation) before and after disinfection for *E. coli* concentration. They found a similar log reduction of *E. coli* at the WWTP using UV disinfection, at a 2.65 log reduction compared to our Site-UV at an average of 1.44 log reduction. The WWTP that used chlorination in their study had a much larger log reduction of *E. coli* in effluent compared to what we observed, at 4.56 log reduction compared to our 2.04 log reduction (Elmund et al., 1999). Osuolale & Okah found similar results when looking at WWTPs in South Africa, documenting over 1 x $10^3$ CFU/100 mL in 58% of samples being released as treated effluent (Osuolale & Okah, 2015). Despite the significant reduction of *E. coli* concentration at both WWTPs ~200 CFU/100 mL are

released in effluent. At Site-UV this could amount to over 431 million CFUs of *E. coli* per day, and at Site-Chlor over 374 million CFUs per *E. coli* per day.

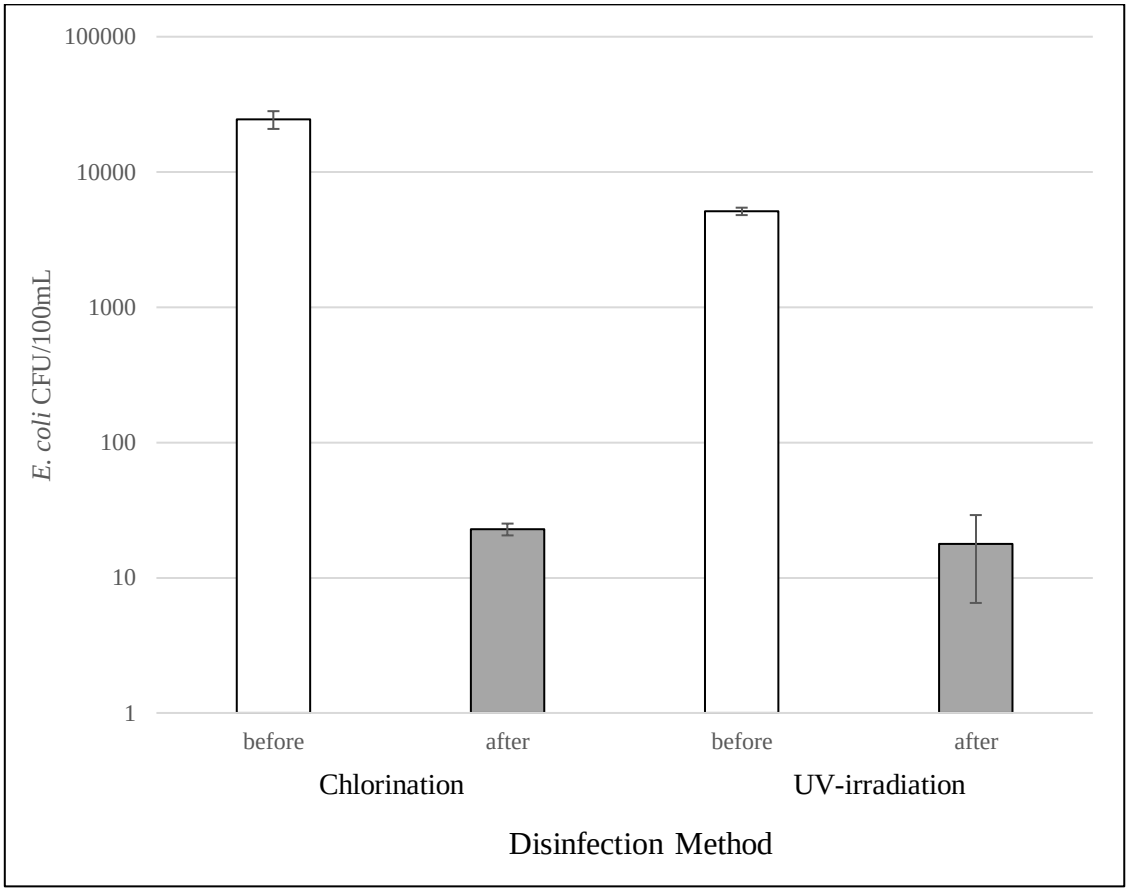


**Figure 5.** *E. coli* concentration pre-and post-disinfection shown as CFU/100 mL, with standard error bars, n = 3.

3.3.2. Phylotypes

At Site-UV, 75 isolates were collected pre-disinfection and 41 post-disinfection, at Site-Chlor 135 isolates were collected pre-disinfection and 143 post-disinfection. Fewer

isolates were collected at Site-UV due to low *E. coli* CFUs present in effluent samples before and after disinfection. Phylotypes seen at Site-UV are presented in Figure 6 as a fraction of total *E. coli* isolates collected. In pre-UV disinfection wastewater (white bars) the most dominant phylotypes were B1, then A, D, C, and B2. The same trend of B1 and A as dominant phylotypes is seen at Site-Chlor in Figure 7. At Site-Chlor after phylotype B1 and A, the next most common phylotypes were C and D, and much less frequently, phylotype B2. Treated effluent from both WWTPs shared B1 and A as their most common phylotypes. We did not expect to see B1 as the most dominant phylotype, as Zhang et al. (2002) documented B2 as the most dominant phylotypes in humans from the United States. However, Zhang took 88 isolates only from healthy women aged 18-39. Perhaps dominant phylotypes differ in males compared to females, and our results reflect that mixture, to date this has not been analyzed. Carlos et al. (2010) also analyzed *E. coli* isolates, but from children aged 1 – 5 years old in Brazil, and found the majority of phylotypes as A (40%), followed by D (38%). There is currently a knowledge gap of dominant *E. coli* phylotypes of healthy males and females in the United States. Also, both Zhang et al. (2002) and Carlos et al. (2010) took isolates obtained directly from the source (rectal), which makes it somewhat difficult to relate to our samples which have undergone wastewater treatment processing before phylotyping. Perhaps B2 is the most common phylotype from pure fecal samples/sources, but B2 may not be able to survive the various stages of wastewater treatment before disinfection as well as phylotypes B1 and A. Furthermore, incoming wastewater may contain fecal material from a variety of sources, not only human.

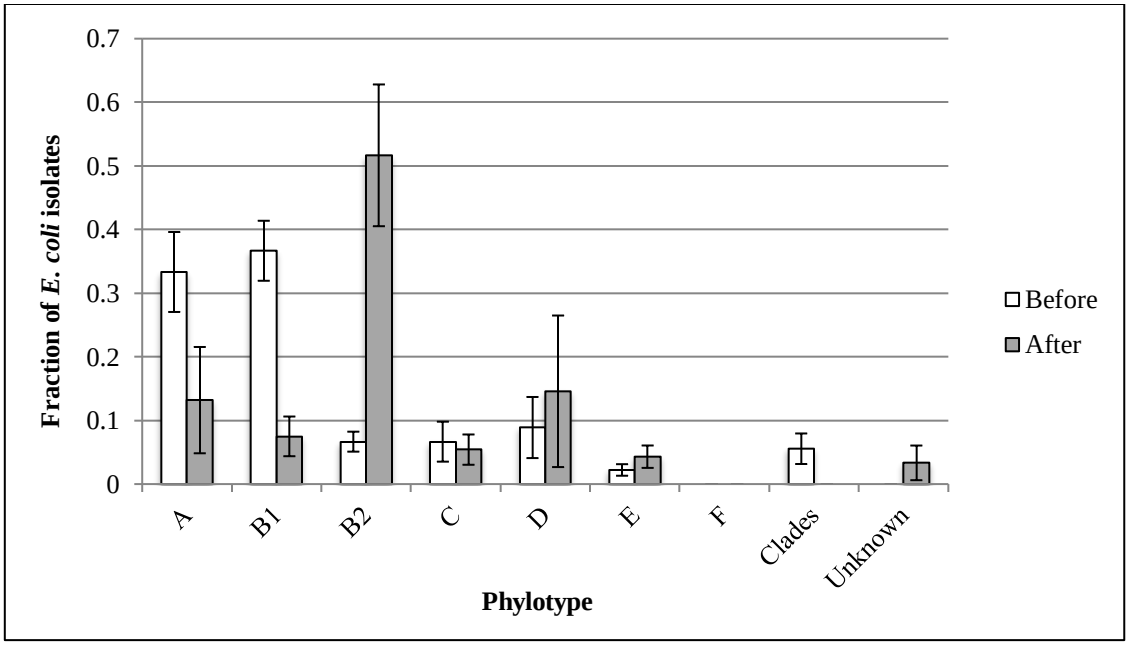


**Figure 6.** *E. coli* phylotypes presented as a fraction of total isolates collected before and after UV disinfection with standard error bars, n = 3.

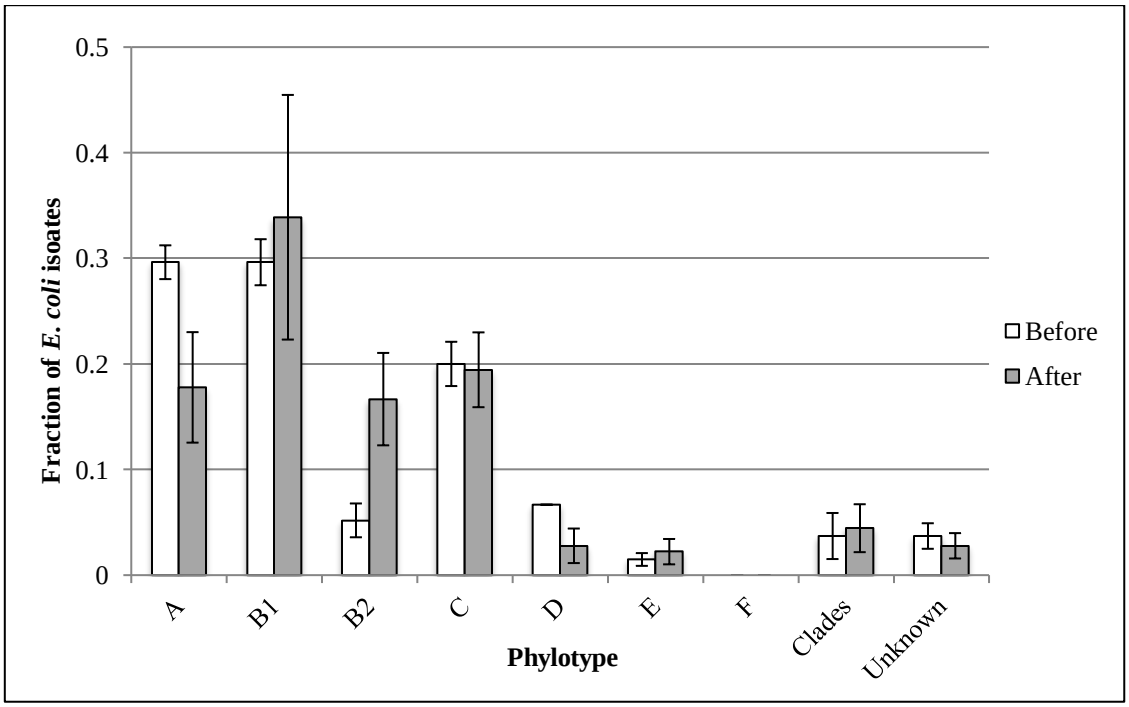


**Figure 7.** *E. coli* phylotypes presented as a fraction of total isolates collected before and after chlorination disinfection with standard error bars, n = 3.

After disinfection, *E. coli* at Site-UV showed a drastic change in dominant phylotype distribution. Instead of B1 as the most dominant phylotype, B2 emerged as the highest in concentration among isolates collected, followed by phylotype D and A, and finally B1. This shift from non-pathogenic phylotypes to potentially pathogenic phylotypes could present an issue for downstream aquatic environments. It's plausible that UV irradiation affects B1 and A phylotypes to a greater extent than B2 and D. Other notable trends at Site-UV include small percentages of phylotype E pre- and post-disinfection. Clades were present pre-disinfection but not post-disinfection, whereas unknown phylotypes were not present pre-disinfection but were present post-disinfection. Phylotype F was completely absent from any sampling date or treatment.

*E. coli* phylotypes after disinfection at Site-Chlor did not experience the same drastic shift in phylotype dominance as at Site-UV. The most common phylotype remained B1, however, the next most common phylotypes were A, B2 & C which were all statistically similar to each other. Phylotype B2 increased in proportion compared to pre-chlorination disinfection, which was almost statistically significant ($p = 0.057$), suggesting B2 may be able to withstand chlorination disinfection better than other phylotypes. Again, phylotype F was not seen in any sampling date or with any treatment. Phylotype E was seen at a small percentage pre- and post-disinfection. Contrary to Site-UV, clades and unknown phylotypes were present pre- and post-disinfection at similar, small percentages.

Frigon et al. (2013) collected samples from four WWTPs (influent after bar screening and prior to disinfection) in Canada and found A was the most dominant

phylotype (40%), followed by B1 (28%) and B2 (23%). Our results agree in that both A and B1 were the most dominant phylotypes from Site-Chlor, and at Site-UV pre-disinfection. Another researcher in Australia, Anastasi et al. (2010) sampled four WWTPs that employed chlorination and found B2 and D were the most dominant phylotypes, accounting for 80% of all phylotypes. However, their methodology included using the triplex-PCR Clermont method instead of the quadruplex-PCR Clermont method which classifies *E. coli* strains into seven phylogroups and two groups of clades instead of only four phylogroups.

Presence of potentially pathogenic phylotypes, B2 and D, in pre- and post-disinfection effluent from both Site-UV and Site-Chlor are presented in Figure 8. Values were calculated using percentage of potentially pathogenic phylotypes present in all samples (pre- or post-disinfection) x *E. coli* colonies present in 100 mL of effluent (pre- or post-disinfection). Potentially pathogenic *E. coli* phylotypes at Site-UV experienced a 2.0 log reduction from pre-disinfection to post-disinfection, and at Site-Chlor a 2.8 log reduction. The larger reduction at Site-Chlor could have been due to a larger initial *E. coli* concentration from pre-disinfection. However, it may also be due to chlorination disinfection being more effective at reducing *E. coli* concentration than UV irradiation. Anastasi et al. (2013) analyzed effluent from a WWTP utilizing chlorination disinfection, and another WWTP utilizing UV irradiation, and suggested that UV irradiation may not be the best disinfection method to eliminate potentially pathogenic *E. coli.* Although the percentage reduction of total *E. coli* in effluent from Site-Chlor and Site-UV was not statistically different ($p = 0.17$) from each other (average 99.9% reduction at Site-Chlor,

99.7% reduction at Site-UV), there was a significant difference in log reduction of total *E. coli* in effluent from Site-Chlor, average 3.1 log reduction, compared to Site-UV, average 2.6 log reduction ($p < 0.002$). Research focusing on *E. coli* reactivation in WWTP effluent after UV disinfection, through both photoreactivation and dark repair, could give a more accurate representation of *E. coli* survival after UV disinfection process.

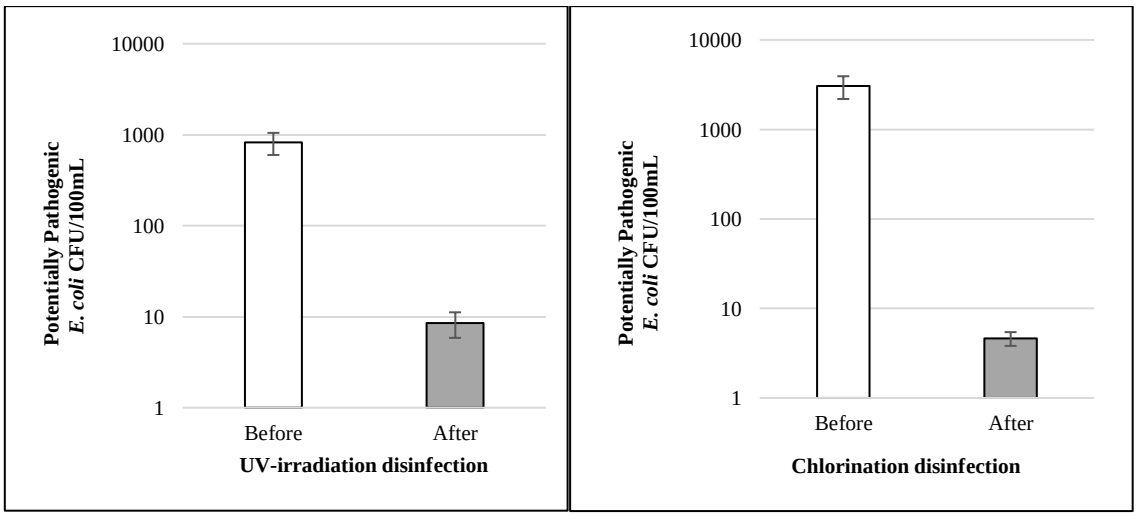


**Figure 8.** Average potentially pathogenic *E. coli* concentration before and after UV and chlorination treatment with standard error bars, n = 3.

Overall phylotype frequency was calculated for each WWTP (Table 5). It's very interesting to note that phylotype B2 is present pre- and post-disinfection at Site-UV and Site-Chlor. It was not the most dominant phylotype through pre- and post-disinfection at each site, but it did demonstrate a constant presence. At Site-UV, the next highest frequency phylotypes were B1 and A, which was almost expected based on their high

proportions in pre and post-disinfection. At Site-Chlor there were four phylotypes present in all treatments and sampling dates: A, B1, B2, and C.

**Table 5.** Frequency of phylotypes seen in all samples and treatments at both WWTPs, presented as a fraction.

| Phylotype | UV WWTP | Chlorination WWTP |
|---|---|---|
| A | 0.83 | 1.00 |
| B1 | 0.83 | 1.00 |
| B2 | 1.00 | 1.00 |
| C | 0.66 | 1.00 |
| D | 0.50 | 0.83 |
| E | 0.66 | 0.66 |
| F | 0.00 | 0.00 |
| Clades | 0.33 | 0.83 |
| Unknown | 0.17 | 0.83 |

3.3.3. Quantitative Microbial Risk Assessment (QMRA) & downstream effects

The quantitative microbial risk assessment for using waters downstream from WWTPs for recreational purposes is shown in Table 6. QMRA calculations show the risk of infection for children swimming once per year or five times per year in an aquatic environment downstream from each WWTP. This calculation does not take into account any *E. coli* naturally present or introduced into the aquatic environment apart from the WWTP effluent. Overall, effluent from Site-UV had higher average yearly levels of estimated risk of infection, if an individual swam only once per year, at 4.0 in 10,000, compared to

effluent from Site-Chlor, at 2.9 in 10,000. If an individual swam five times in a one-year period the level of estimated risk for swimming downstream from Site-UV rose to 26.7 in 10,000, and downstream from Site-Chlor 19.7 in 10,000. Comparing QMRA from both Site-Chlor and Site-UV showed there was no statistical difference of risk between effluent from Site-Chlor and Site-UV for one swimming event per year ($p = 0.47$) or five swimming events per year ($p = 0.46$). This is due to higher percentages of B2 phylotypes present in effluent water treated with UV irradiation. *E. coli* levels could be even higher in downstream communities from UV WWTPs, as some *E. coli* cells are capable of photoreactivation and nucleotide excision repair to repair damage done by UV rays (Quek & Hu, 2008). Further research using QMRA and assessing regrowth after UV treatment could provide broader analysis of the effects of *E. coli* downstream from WWTPs.

**Table 6.** Estimated infection rate per 10,000 swimming/recreational activities, calculated using number of times (once or five times) per year individual swims in downstream effluent.

| Treatment | Once per year | Five times per year |
|---|---|---|
| UV-1 | 3 | 20 |
| UV-2 | 5 | 33 |
| UV-3 | 4 | 27 |
| UV-Average | 4 | 26.7 |
| | | |
| Chlor-1 | 0.8 | 6 |
| Chlor-2 | 5 | 33 |
| Chlor-3 | 3 | 20 |
| Chlor-Average | 2.9 | 19.7 |

Another effect on downstream environments on a microbial scale is that WWTP effluent may be reducing natural variability and creating a biotic homogenization (Drury et al., 2013). Drury et al. (2013) sampled stream sediment downstream from two WWTPs in Chicago, IL and found that the influx of WWTP effluent significantly altered sediment bacterial communities by decreasing both abundance and diversity. An influx of WWTP effluent, including *E. coli*, could be altering stream microbial communities.

### 3.4. Conclusion

Chlorination and UV- disinfection both significantly reduced total *E. coli* concentration, as expected. Phylotype results from this study indicate that the most dominant phylotypes from WWTPs before disinfection are A and B1 at both WWTPs. Previously, B2 was thought to be the dominant phylotype from human *E. coli*, our research indicates B1 and A may be more prominent pre-disinfection, or that B1 and A may be better able to survive WWTP treatment processes prior to disinfection. Higher concentrations of potentially pathogenic phylotypes were found in the WWTP that employed UV-irradiation. QMRAs conducted on effluent from both WWTPs showed no statistical difference between disinfection method, however, risk from using downstream waters for recreational purposes are elevated compared to natural *E. coli* levels. As of 2016, there are over 16,000 publicly owned wastewater treatment facilities in the United States (USDHS, 2016). These results can help water and wastewater stewards better predict the effect treated effluent has on downstream aquatic environments, especially if they are used for any recreational purposes.

# CHAPTER IV

# PHYLOTYPE DISTRIBUTION OF *E. COLI* DURING PHOTOREACTIVATION AND DARK REPAIR IN WASTEWATER TREATMENT PLANT EFFLUENT AFTER ULTRAVIOLET DISINFECTION

## 4.1. Introduction

Wastewater treatment plants (WWTPs) typically employ either chlorination or Ultra Violet (UV) irradiation as their disinfection step to eliminate microorganisms from effluent. Chlorine disinfection can be more cost effective than UV irradiation, unless dechlorination is required, but can release chlorine residuals which can be toxic to downstream environments (EPA, 1999). UV irradiation has become a popular alternative in recent years (EPA, 1999), and has comparable microorganism reduction levels, as seen in various studies (Chang et al., 1985; Watts et al., 1995; Lazarova et al., 1999; Koivunen et al., 2005). UV irradiation operates by using UV light at 254 nm, as it creates lesions in microbial DNA, termed pyrimidine dimers (Harm, 1980; Friedberg et al., 1995). The pyrimidine dimers prevent future translation and replication and thus inactivates the microorganism.

However, several microorganisms with UV induced pyrimidine dimers are able to repair the damage using light or dark repair mechanisms. Light repair, also known as photoreactivation, is light-dependent and requires the activation of the enzyme photolyase. Photolyase locates the pyrimidine dimer, binds to the DNA, then with natural light at 345-400 nm wavelength photolyase changes its configuration and breaks apart the pyrimidine

dimer (Walker, 1984; Oguma et al., 2001). The microorganism's DNA is returned to its original state and can replicate. Dark repair after UV damage, also known as nucleotide excision repair, requires the coordination of a dozen different enzymes, as well as energy supplied by nutrients within the cell. Dozens of enzymes activated by the microbe create a complex that excises a large section of DNA surrounding the pyrimidine dimer (Quek & Hu, 2008). Afterwards, DNA on the excised region is replaced by complementary bases from the unexcised region opposite by DNA polymerase and DNA ligase (Quek & Hu, 2008).

*Escherichia coli* (*E. coli*) is a microorganism commonly found in human and animal waste, originating from warm blooded mammals. Certain strains of *E. coli* are capable of causing infection and/or illness, such as *E. coli* O157:H7. Additionally, *E. coli* is capable of photoreactivation and dark repair following UV-inactivation (Masschelein, 2003; Sanz et al., 2007). There are various classification methods for strains of *E. coli*, one of the most recently used is phylotyping. Phylotype classification can give insight into origin host, pathogenicity, and preferred habitat (Alm and Walk, 2011). Current phylotype classification involves the screening for three genes (*arpA, trpA, yjaA*) and a DNA fragment (TspE4.C2) to place a strain in a phylogroup (A, B1, B2, C, D, E, F, clades I-V) (Clermont et al., 2013). Phylogroups which may contain pathogenic *E. coli* strains include B2 and D, both are considered potentially pathogenic phylotypes. Elucidating *E. coli* phylotype distribution could give insight into potential pathogenicity of *E. coli* present in a water sample.

Photoreactivation and dark repair of *E. coli* pose a problem for the efficacy of using UV-irradiation for disinfection at WWTPs (Sanz et al., 2007). Treated WWTP effluent is often used for agricultural purposes, landscape irrigation and golf course irrigation (EPA, 2016). Treated effluent from WWTPs utilizing UV irradiation is released downstream and is subject to environmental conditions that may be conducive to photoreactivation and/or dark repair. Many studies have found *E. coli* capable of both light and dark repair mechanisms after UV disinfection (Kaur et al., 2016; Childress et al., 2014; Quek & Hu, 2008; Sanz et al., 2007; Masschelein, 2002; Zimmer & Slawson, 2002; Harris et al., 1987). The majority of recent studies have focused on the relationship between UV dose and *E. coli* repair rates (Guo et al., 2009; Sanz et al., 2007; Zimmer & Slawson, 2002: Oguma et al., 2002), and few have analyzed samples directly from WWTPs (Anastasi et al., 2013; Anastasi et al., 2010). No studies to date have analyzed *E. coli* phylotypes from UV treated WWTP effluent subsequently stored in dark and light conditions.

In 2014, Texas Commission on Environmental Quality issued a surface water quality report and identified water segments that do not currently meet water quality standards and placed them on the 303 (d) List of Impaired Waters (TCEQ, 2014). Water segments located downstream from a WWTP in central Texas have been on the 303 (d) List of Impaired Waters since 1999 due to *E. coli* contamination (TCEQ, 2014). Collecting UV irradiated effluent directly from an operating WWTP gives a more accurate analysis of *E. coli* concentration and *E. coli* phylotype distribution released downstream, compared to studies that use autoclaved wastewater with introduced *E. coli* strains. The objectives of this study were to analyze *E. coli* concentration in effluent collected after

UV disinfection, investigate photoreactivation and dark repair rates, and determine any trends of potentially pathogenic and non-potentially pathogenic *E. coli* phylotypes with respect to photoreactivation and dark repair mechanisms.

### 4.2. Materials and methods

Effluent samples were taken pre- and post-UV disinfection from a WWTP in central Texas on March 2, May 4, and May 18, 2015 in 1-L Pyrex bottles and transported back to the laboratory. Pre-disinfection samples were processed according to EPA Method 1603 (modified mTEC) for enumerating *E. coli* (EPA, 2002). Filtered samples were incubated at 35°C ± 0.5°C for 2 hrs, then incubated at 44.5°C ± 0.2°C for 22 hrs. After 24 hrs mTEC plates (Nasco, Modesto, California) were counted and red-magenta colonies were considered *E. coli* colonies. Up to ten *E. coli* colonies were randomly selected and isolated from each mTEC plate and transferred onto EC-MUG medium (EMD, Gobbstown, NJ) as a secondary screen for β-glucuronidase enzyme activity. After 24 hrs incubation, colonies were isolated and stored in tryptic soy broth with 20% glycerol at -80°C for future polymerase chain reaction (PCR) analysis. Post-disinfection samples in 1-L Pyrex bottles were stored in light conditions (left in ambient light conditions) or dark conditions (bottle covered with aluminum foil to prevent any light penetration). Both treatments were incubated on a magnetic stir plate at 125 rpm and room temperature (~21°C). Pyrex bottles were randomly distributed on the stirrer and randomly rearranged at each sampling time. One 100 mL aliquot was taken from each of the three replicates from each treatment

(light or dark) at 0 hr, 12 hrs, 24 hrs and 48 hrs, and *E. coli* was enumerated with EPA Method 1603 (modified mTEC, as mentioned above).

Phylotyping of *E. coli* colonies involved isolating colonies from EC-MUG plates and storing them individually in 0.1 mL aliquots of molecular grade sterile water (cell suspensions) at -20°C. PCR reactions included 12.5µL of GoTaq G2 Green Master Mix, 1.5 µg/µL bovine serum albumin, 4.5 µL of molecular grade $H_2O$, 5 µL of DNA template (cell suspension), 1 µL of forward primer and 1 µL of reverse primer at a concentration of 20 pmol/µL (see Table 1). PCR conditions (described by Clermont et al. 2013) included denaturation at 94°C for 10 min; followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec; final elongation at 72°C for 7 minutes; and holding at 4°C until removed from the Eppendorf Mastercycler (Hamburg, Germany). Gel electrophoresis was used to visualize PCR products using a 2% agarose gel prepared with 1 x TBE buffer and 0.5 µg/mL ethidium bromide. DNA ladders were used (exACT Gene Low Range Plus DNA Ladder) in the first and last well of each gel for base pair length comparison and determination along with positive and negative controls for each gene segment analyzed. Electrophoresis occurred at 125V for 25-35 minutes. Gels were imaged with an UltraLum Omega Molecular Imaging System (Carlsbad, CA). A second PCR reaction was conducted on colonies which had the classification of A or C, or E or D; C and E primers were used to finalize phylotype classification.

4.2.1. Survival ratio, repair percentage and hourly repair rate

As a measure of reactivation, survival ratio values were calculated using an equation from Sanz et al. (2007):

$$S_t = \frac{N_t}{N_0} \times 100$$

Where $S_t$ is the survival ratio at time *t*, $N_t$ is the *E. coli* concentration at time *t* (CFU/100 mL), and $N_0$ is the *E. coli* concentration before disinfection (CFU/100 mL).

Percentage repair was measured using a formula from Quek and Hu (2008), previously altered from Lindenauer and Darby (1994). Quek and Hu log-transformed *E. coli* concentrations in the equation, thereby normalizing the concentrations to express them as repair percentage. This allows the comparison of light and dark repair, regardless of their initial concentration.

$$\%\ repair = \frac{N_t - N_0}{N_{initial} - N_0} \times 100$$

Where $N_t$ is the log concentration (CFU/100mL) of *E. coli* at time *t,* $N_0$ is the log concentration of *E. coli* immediately after disinfection, and $N_{initial}$ is the log concentration (CFU/100mL) of *E. coli* before disinfection (collected at time of sampling).

Repair rate is calculated using an equation adapted from Quek and Hu (2008):

$$Rate\ of\ repair = \frac{N_t - N_0}{t}$$

Where $N_t$ is the concentration of *E. coli* (log CFU/100 mL) after *t* hours, $N_0$ is the concentration of *E. coli* (log CFU/100 mL) immediately after disinfection, and *t* is the time interval between samples (either 12 or 24 hours in this study).

4.2.2. Statistical analyses

Means of all treatments were compared in Excel using a two-tailed t-test of all three or nine runs, with statistical significance identified with a p-value $\leq 0.05$.

## 4.3. Results and discussion

4.3.1. *E. coli* concentration

For the March 2 sampling date, *E. coli* concentrations in both light and dark treatments were not statistically different from each other at any time point (Figure 9). At 48 hrs, however, the average for the light treatment had a higher concentration than the dark treatment, at 735 CFU/100 mL compared to 276 CFU/100 mL. *E. coli* average concentrations from samples taken on May 4, 2015 were higher at 12, 24, and 48 hrs in the light treatment than the dark treatment, however, only at 48 hrs were the concentrations statistically significant ($p = 0.01$) (Figure 10). *E. coli* concentration on the last sampling date, May 18, 2015, showed higher averages for *E. coli* concentrations in the light treatment, however, it was not statistically significant for any time point (Figure 11). We combined the sampling dates to present a graph of *E. coli* concentration after UV irradiation in dark and light treatments for spring 2015 (see Figure 12). At time 0 hrs,

immediately after UV disinfection, *E. coli* concentration for both treatments were equal, as this is before effluent is divided into separate treatments. We expected to see initial *E. coli* concentrations between 0 and 50 CFU/100 mL, as that has been previously recorded at this WWTP after UV disinfection (Childress et al., 2014). We saw similar values as Childress et al. (2014), averaging around 28 CFU/100 mL. At 12 and 48 hrs *E. coli* in light treatment reached a higher concentration and is statistically significant (12 hrs $p = 0.05$; 48 hrs $p = 0.01$). However, at 24 hrs *E. coli* concentrations in light and dark treatments were not statistically different from each other. In light treatment both photoreactivation and dark repair may be occurring simultaneously; this is because photoreactivation requires a light source and dark repair does not. Dark repair does not necessarily require dark conditions, simply the coordination of dozens of enzymes to excise the affected DNA region and replace it with complimentary bases. In the dark treatment only dark repair can occur as all light is theoretically prevented from penetrating the Pyrex bottle. The trend of higher *E. coli* concentrations in the light treatment compared to the dark treatment has been previously documented by Childress et al. (2014) as well. Childress took UV-treated effluent from the same WWTP used in this study and exposed the effluent to light or dark conditions for 9 hrs and found *E. coli* concentrations reached a maximum of 190 CFU/100 mL at 4 hrs in the light treatment and 160 CFU/100 mL at 2 hrs in the dark treatment (Childress et al., 2014). Zimmer & Slawson (2002) sampled drinking water for *E. coli* after treating samples with low and medium pressure UV lamps and storing them in light and dark conditions, they also found higher concentrations of *E. coli* in light compared to dark treatment.

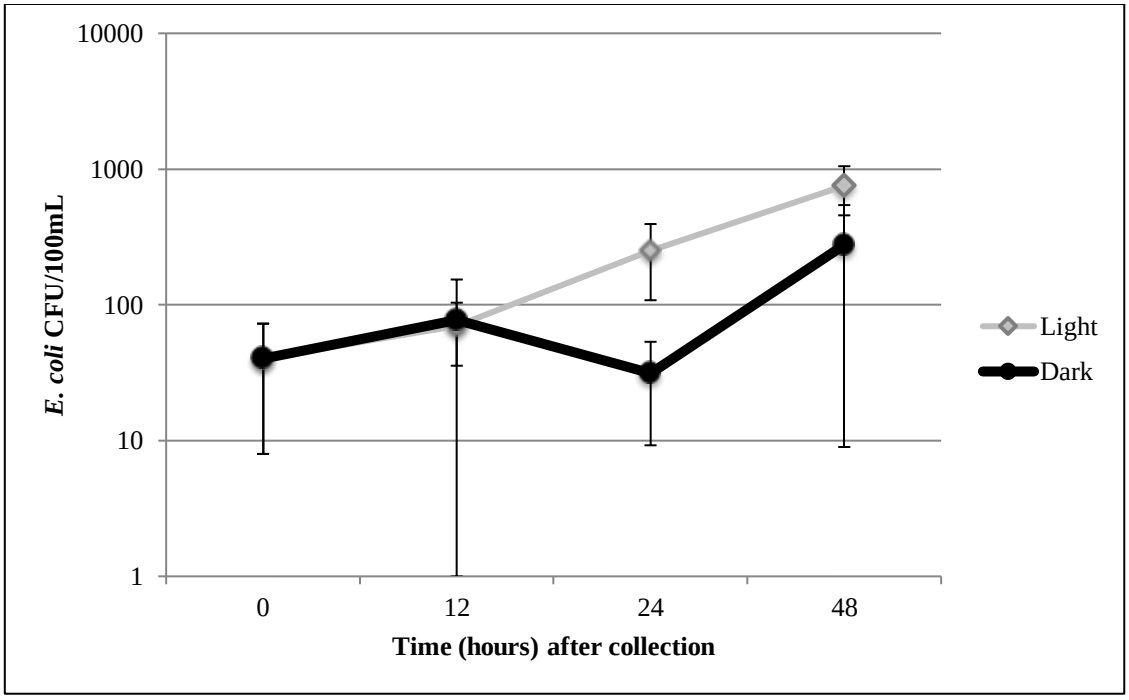


**Figure 9.** *E. coli* concentration for first sampling date, March 2nd, 2015 in light and dark treatment (n = 3) with standard error bars. Samples in light treatment were kept in Pyrex bottles exposed to ambient light conditions and kept at room temperature (~ 21°C). Samples in dark treatment were kept in Pyrex bottles with aluminum foil to prevent any light penetration, kept at room temperature (~ 21°C).

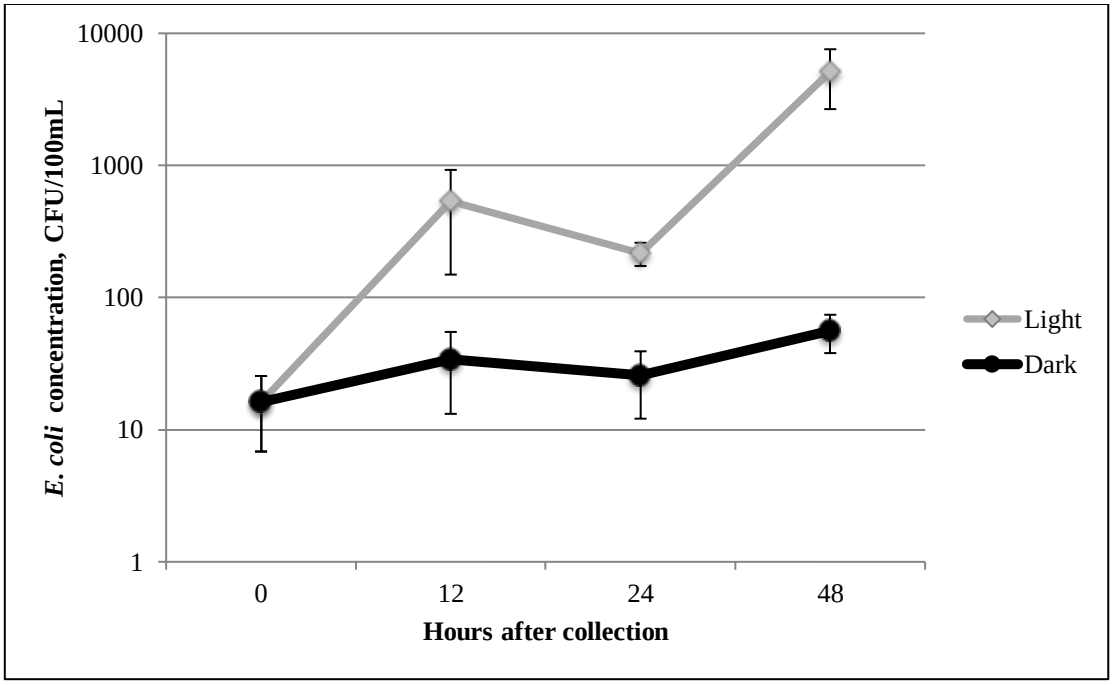


**Figure 10.** *E. coli* concentration for second sampling date, May 4th, 2015 in light and dark treatment (n = 3) with standard error bars. Samples in light treatment were kept in Pyrex bottles exposed to ambient light conditions and kept at room temperature (~ 21°C). Samples in dark treatment were kept in Pyrex bottles with aluminum foil to prevent any light penetration, kept at room temperature (~ 21°C).

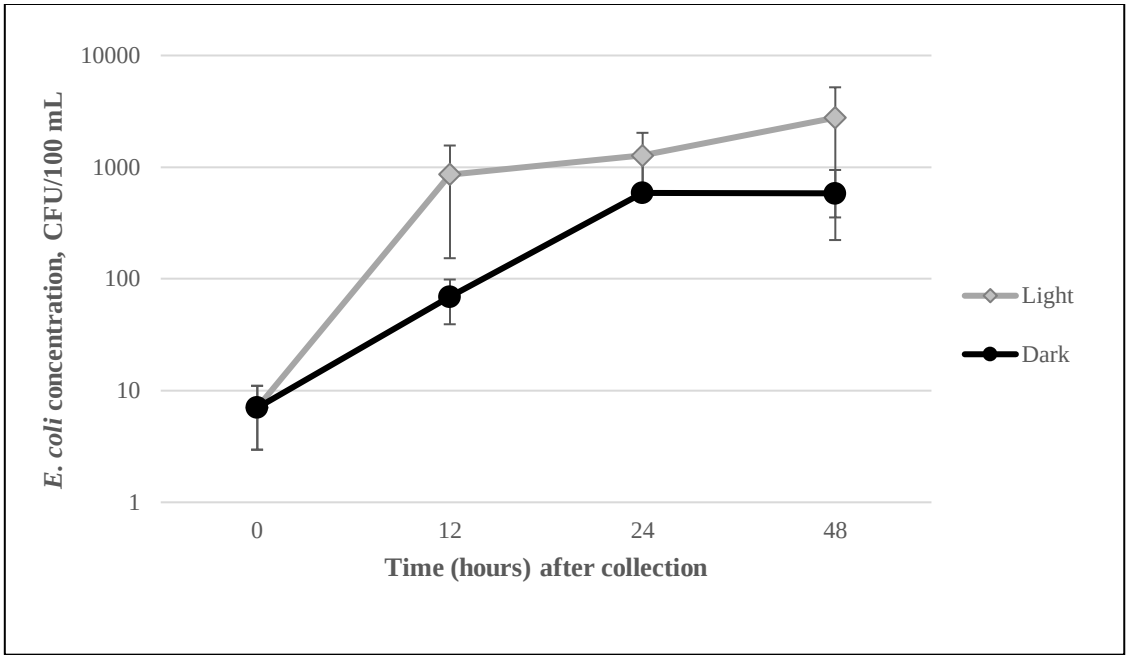


**Figure 11.** *E. coli* concentration for second sampling date, May 18, 2015 in light and dark treatment (n = 3) with standard error bars. Samples in light treatment were kept in Pyrex bottles exposed to ambient light conditions and kept at room temperature (~ 21°C). Samples in dark treatment were kept in Pyrex bottles with aluminum foil to prevent any light penetration, kept at room temperature (~ 21°C).

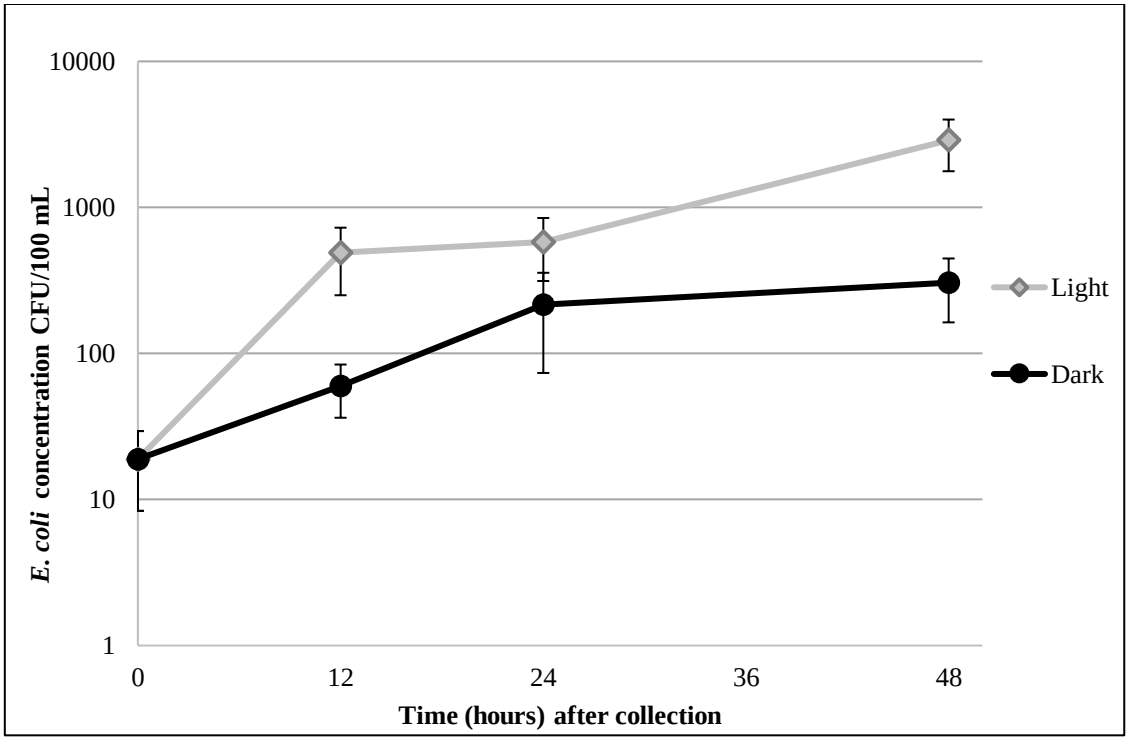


**Figure 12.** *E. coli* concentration for all sampling dates combined for light and dark treatment (n = 9) with standard error bars. Samples in light treatment were kept in Pyrex bottles exposed to ambient light conditions and kept at room temperature (~ 21°C). Samples in dark treatment were kept in Pyrex bottles with aluminum foil to prevent any light penetration, kept at room temperature (~ 21°C).

4.3.2. Survival ratio

Survival ratios varied from 0.14 to 0.37% immediately after disinfection, with an average of 0.23% (Figure 13). Average survival values for the light treatment were 12.9% at 12 hrs, 13.8% at 24 hrs, and 75.8% at 48 hrs. Average survival values for the dark treatment were 1.1% at 12 hrs, 5.4% at 24 hrs, and 6.2% at 48 hrs. Survival values at 12, 24 & 48 hrs may include regrowth from *E. coli* cells. *E. coli* cells that survived the initial disinfection process most likely continued to replicate and that population is included in the survival ratio calculation (instead of eliminating the effect). Survival ratio values were

higher for *E. coli* in effluent kept in the light treatment, and statistically significant at 12 and 48 hrs at the $p < 0.05$ level. The opposite trend was recorded by Childress et al. (2014), they documented similar survival rates of *E. coli* kept in dark conditions (up to 100% at 4 hrs) compared to light conditions (up to 100% at 2 hrs). They suggested that the higher levels of dark repair they observed could have been due to the type of *E. coli* studied (wild-type versus laboratory strain). While that may be true, Chan and Killick (1995) isolated a wild *E. coli* strain and after subjecting water samples with *E. coli* to UV-irradiation and storing them in light and dark conditions, found a maximum recovery of 52% at 6 hrs in the light, and 22% at 9 hrs in the dark. Although we didn't observe a comparable high level of survival in dark conditions, our results agree more with Chan and Killick's results, even though we used larger time intervals. It's possible that due to larger intervals between sampling times we may have missed peak survival ratios from an exponential growth phase between 0 and 12 hrs. Sanz et al. modeled fecal coliforms in unfiltered wastewater effluent treated with varying levels of UV-C doses (50-200 m W s/cm$^2$) and recorded survival ratios up to 2.3%, which is much lower than our values. However, Sanz et al. (2007) only enumerated fecal coliforms up to 4 hrs, it's possible that survival ratios could have increased between 4 and 12 hrs. Survival ratios between 0 – 4 hrs are very informative, however, we sought to understand longer-term survivability of *E. coli* after release from WWTPs.

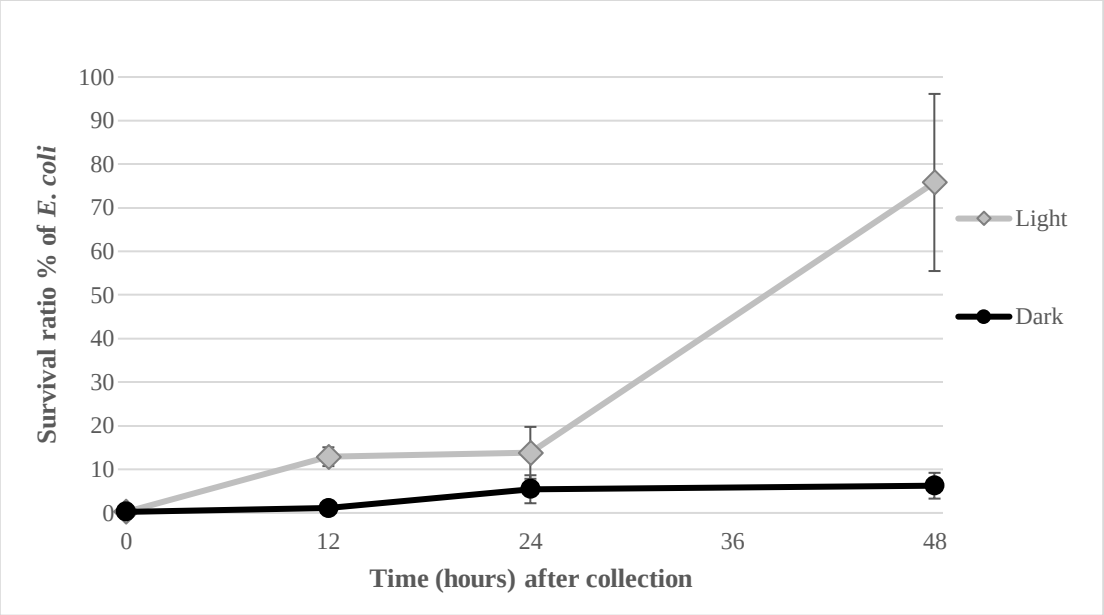


**Figure 13.** Survival ratio for *E. coli* cells in light and dark treatment for all sampling dates combined (n = 9) with standard error bars. Samples in light treatment were kept in Pyrex bottles exposed to ambient light conditions and kept at room temperature (~ 21°C). Samples in dark treatment were kept in Pyrex bottles with aluminum foil to prevent any light penetration, kept at room temperature (~ 21°C).

4.3.3. Percentage repair

Figure 14 shows the repair percentage for both light and dark treatments. The average repair percentage for *E. coli* in the light treatments starts at 40.8% at 12 hrs, slightly increases to 45.3% at 24 hrs, and reaches 70.1% at 48 hrs. In the dark treatment, *E. coli*'s repair rate starts out negatively at -15.9% at 12 hrs, moves to 2.4% at 24 hrs, and reaches 15.1% at 48 hrs. We expected to see higher rates of repair for *E. coli* in the light treatment, in that *E. coli* may undergo both photoreactivation and dark repair simultaneously. The negative repair rate seen with dark repair of *E. coli* at 12 hrs was the result of *E. coli* concentration lower at 12 hrs compared to immediately after UV disinfection. This could

have been due to a variety of factors, most likely low rates of nucleotide excision repair coupled with a possible die-off stage of *E. coli* at that time, perhaps due to adapting to a new, dark environment. Quek & Hu noted their highest *E. coli* dark repair rate after only 2 hrs and suggested that their *E. coli* populations may have reached a plateau afterwards (Quek & Hu, 2008). Our study showed a higher repair percentage reached at 48 hrs at 15.1%, however, Quek & Hu's study did not reach to 48 hrs.

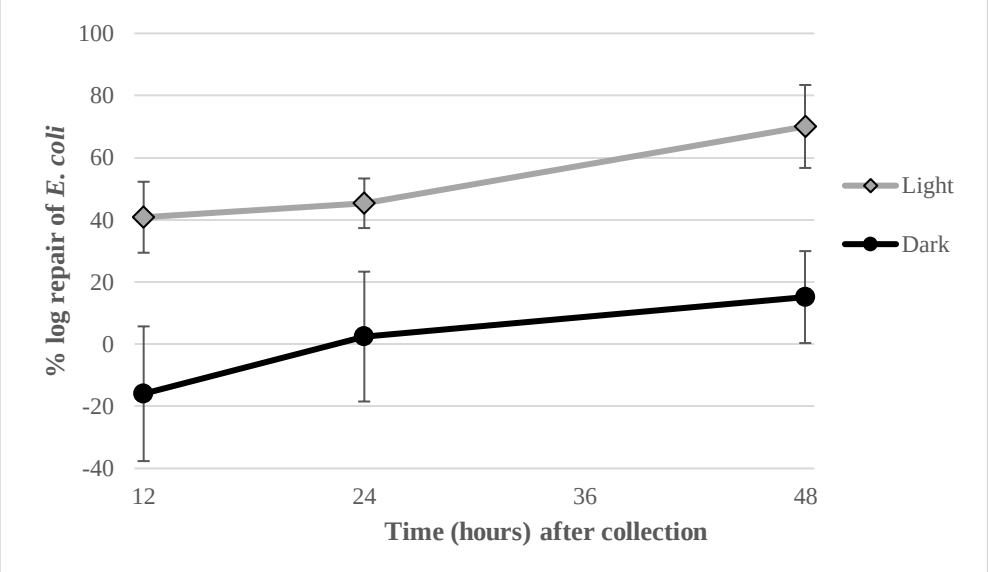


**Figure 14.** *E. coli* log repair (%) in light and dark treatment for all sampling dates combined (n = 9) with standard error bars. Samples in light treatment were kept in Pyrex bottles exposed to ambient light conditions and kept at room temperature (~ 21°C). Samples in dark treatment were kept in Pyrex bottles with aluminum foil to prevent any light penetration, kept at room temperature (~ 21°C).

4.3.4. Repair rate

Repair rates were measured between 0 and 12 hrs, 12 and 24 hrs, and 24 and 48hrs. Repair rates in the light treatment were considerably higher than in the dark treatment, with the highest average for both seen at 24 hrs (light = 0.13; dark = 0.06) (Figure 15). Quek and Hu (2008) reported much higher rate of repair for their *E. coli* strains, from 0.2 to 3.3, however they sampled concentration on an hourly basis, while we used 12 and 24 hr time intervals and may have missed large population increases in the first 2-6 hrs of growth (Quek and Hu, 2008).

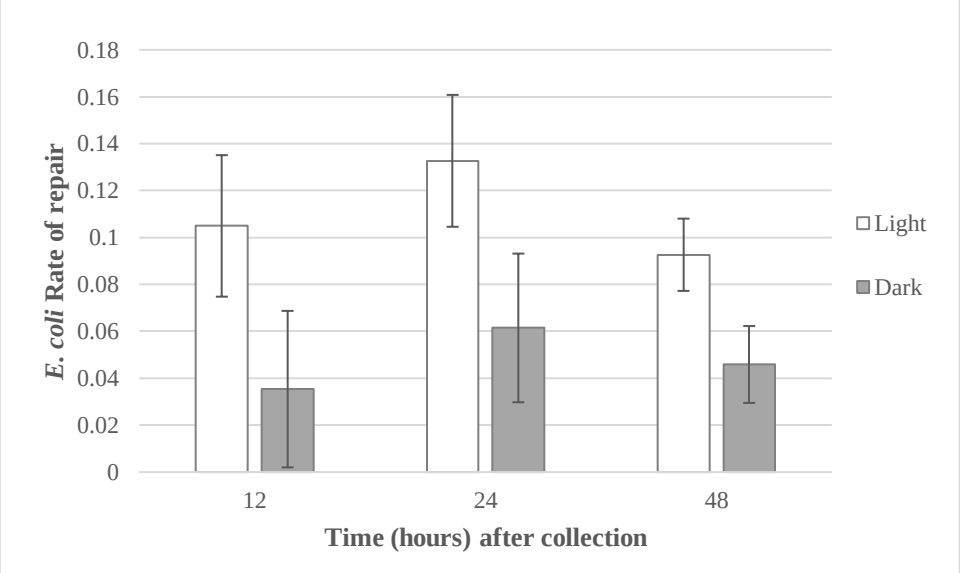


**Figure 15.** Rate of repair for *E. coli* in light and dark treatment over sampling period of 48 hrs with standard error bars (n = 9). Samples in light treatment were kept in Pyrex bottles exposed to ambient light conditions and kept at room temperature (~ 21°C). Samples in dark treatment were kept in Pyrex bottles with aluminum foil to prevent any light penetration, kept at room temperature (~ 21°C).

Other environmental factors such as salinity and temperature could have an effect on *E. coli* reactivation rates. Chan and Killick (1995) subjected *E. coli* to varying levels of salinity at 15°C over a 1 hr period and found that a salt concentration above 30% significantly lowered *E. coli*'s ability to photoreactivate. They also added 0.25% glucose to water samples with *E. coli* in dark conditions to see if it would increase dark repair rates, however it did not, suggesting that a lack of nutrients may not be the cause of low dark repair rates (compared to photoreactivation rates) (Chan and Killick, 1995). Kollu and Örmeci (2014) further confirmed this using *E. coli* ATCC 23631, subjecting *E. coli* to UV-irradiation, and analyzing regrowth in phosphate-buffered saline (PBS) solution, nutrient-enriched PBS, and autoclaved wastewater. They found significant regrowth of *E. coli* in the PBS solution, indicating that cells previously lysed could be providing a source of nutrients for new cell development. Xu et al. (2015) used *E. coli* strain AB2463, exposed it to UV-irradiation, kept samples at varying temperatures and studied the photoreactivation rates. They found that temperatures higher than 37°C decreased photolyase proteins in *E. coli* cells; using assay activity and *in vitro* differential scanning calorimetry showed denaturation of photolyase above 37°C (Xu et al., 2015). Shafaei et al. (2016) also analyzed the effect of temperature on photoreactivation rates of coliforms and found the highest photoreactivation rate (90%) occurred at 25°C, from WWTP effluent from Alberta after exposure to UV-irradiation. This leads to the conclusion that ideal temperature for *E. coli* photoreactivation may occur between 25°C - 36°C. We kept samples at room temperature (~ 21°C), we may have missed peak photoreactivation rates.

4.3.5. *E. coli* phylotype distribution after UV disinfection

The most common *E. coli* phylotypes are seen in Figure 16a for light treatment and Figure 16b for dark treatment. *E. coli* phylotype percentages are calculated as number of *E. coli* isolates in each phylogroup divided by the total number of isolates collected. The most dominant phylotype for *E. coli* in the light treatment was B2 at 0 hrs, coming in at 51.7% immediately after disinfection. Both A and B1 phylotypes are under 15% representation at 0 hrs, A at 13.3% and B1 at 7.5%. At 12 hrs the phylotype dynamics change, B2 drops immensely down to 21.9%, B1 rises to 17.3%, and A reaches 30.3%. At 24 hrs this trend continues for A at 30.8%, B1 at 20%, and B2 continues its drop to 6.7%. However, at 48 hrs B2 regains its position as the dominant phylotype at 31.6%, B1 rises to 29.2%, and A drops down to 18.1%. Phylotype B2 may have had highly variable representation throughout the entirety of the incubation period, however it remained one of the top three dominant phylotypes for the duration of the experiment. Phylotype B1 steadily rose from 0 to 12, 12 to 24, and 24 to 48 hrs. It's possible that B1 is very susceptible to UV damage initially, but may benefit from photoreaction more so than other phylotypes.

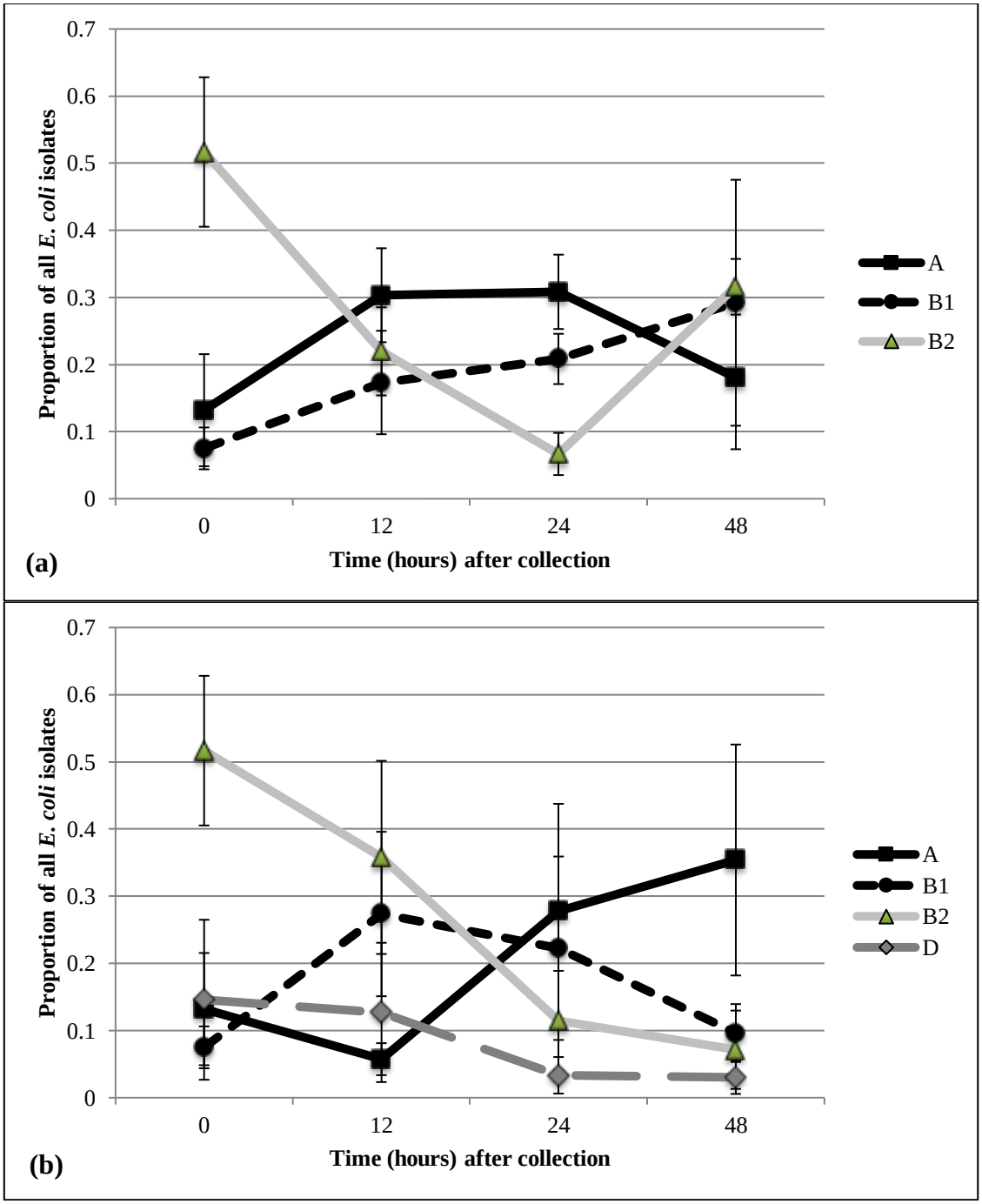


**Figure 16.** Dominant *E. coli* phylotypes presented as a fraction of all phylotypes in light treatment (a) and dark treatment (b) with standard error bars (n = 3). Samples in light treatment were kept in Pyrex bottles exposed to ambient light conditions and kept at room temperature (~ 21°C). Samples in dark treatment were kept in Pyrex bottles with aluminum foil to prevent any light penetration, kept at room temperature (~ 21°C).

The most common phylotypes in the dark treatment are shown in Figure 3b. The initial (time 0 hrs) phylotype distribution is the same as for light treatment, however we have included phylotype D as it remained a significant proportion of phylotypes throughout the sampling period. The two potentially pathogenic phylotypes (B2 and D) represent almost 66.3% of all *E. coli* phylotypes at time 0 hrs. At 12 hrs, phylotype B2 and D both decrease to 35.8% and 12.7%, respectively, along with phylotype A at 5.7%. Phylotype B1 rose significantly to 27.4%. From 12 to 24 hrs phylotypes B1, B2, and D all decreased to 22.2%, 11.5%, and 3.3.%, respectively. Phylotype A is the only phylotype to increase in percentage to 27.8% at 24 hrs, and it continues this increase up to 35.4% at 48 hrs, while B1, B2 and D continue their decline to 9.6%, 7.1%, and 3.1%, respectively. For phylotype A, its progression through the sampling period somewhat resembled a growth curve. The fact that *E. coli* phylotypes differ in their photoreactivation and repair rates may indicate that certain phylotypes may have mechanisms, genes, or traits that help them adapt to dark or light conditions, or survive in nutrient poor or very competitive environments. Sommer et al. (2000) analyzed photoreactivation of *E. coli* after UV inactivation and found that different strains of *E. coli* experienced different photoreactivation rates. It is possible that each separate *E. coli* phylotype could have varying photoreactivation or dark repair rates, no studies to date have analyzed this.

4.3.6. Potentially pathogenic phylotypes

The potentially pathogenic phylotype distribution in dark and light treatments is presented in Figure 17. At time 0 hrs, UV-irradiated treated effluent had a very high percentage of

potentially pathogenic phylotypes (B2 and D) at 66.3%. Following *E. coli* potentially pathogenic phylotypes in the dark treatment, the percentage decreased from 0 to 12 hrs (48.5%), 12 to 24 hrs (14.8%), and 24 to 48 hrs (10.2%). This may indicate that potentially pathogenic phylotypes are not able to compete as well as their non-pathogenic phylotype counterparts (A, B1, C, E, F, etc) in dark environments. Switching over to potentially pathogenic phylotypes in the light treatment, they decrease dramatically from 0 to 12 hrs (23.1%), to a greater extent than seen in the dark treatment. It slightly decreases again from 12 to 24 hrs (17.5%), and then increases from 24 to 48 hrs (29.6%). As this increase of potentially pathogenic phylotypes was not seen at 48 hrs in the dark treatment, it is possible that there may be an adaptive advantage of potentially pathogenic phylotypes in light conditions over a longer period of time. However, there was no statistical difference between potentially pathogenic phylotypes in light and dark treatments.

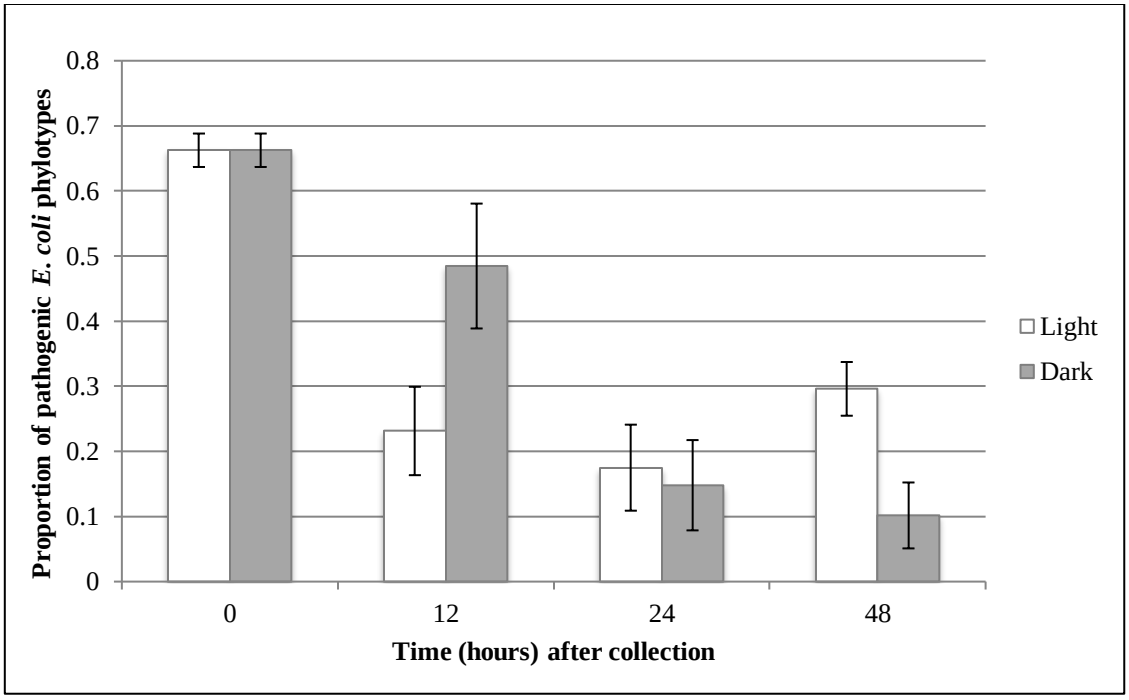


**Figure 17.** Fraction of potentially pathogenic *E. coli* phylotypes for both light and dark treatment, with standard error bars (n = 3). Samples in light treatment were kept in Pyrex bottles exposed to ambient light conditions and kept at room temperature (~ 21°C). Samples in dark treatment were kept in Pyrex bottles with aluminum foil to prevent any light penetration, kept at room temperature (~ 21°C).

*E. coli* exhibits genome plasticity, questions have arose concerning pathogenic *E. coli*'s ability to retain pathogenic characteristics through UV-irradiation and subsequent photoreactivation. Guo et al. (2012) examined *E. coli*'s ability to retain certain characteristics, namely ampicillin resistance, through UV-irradiation and photoreactivation and discovered that ampicillin resistance was easily retained, and *E. coli* with ampicillin resistance exhibited similar photoreactivation rates as *E. coli* without ampicillin resistance.

Hallmich and Gehr (2010) researched the effect environmental conditions, such as access to light, had on photoreactivation of fecal coliforms, from WWTP effluent in Quebec, and found that if UV treated water is kept away from photoreactivating light for 3 hours before release, the subsequent photoreactivation recovery percentages were significantly lower than samples immediately subjected to light conditions. They continued a step further and exposed their samples to visible light (for 2-3 min) prior to UV-irradiation and found that *E. coli* photoreactivation recovery percentage was lowered by 46 ± 13% when compared to samples not previously exposed to visible light. Even more surprising was when visible light and UV-irradiation occurred simultaneously, they observed an *E. coli* photoreactivation reduction of 56 ± 13% compared to UV-irradiation alone (Hallmich & Gehr, 2010). This suggests that simultaneously exposing WWTP effluent to both visible light and UV-irradiation, possibly by conducting UV-irradiation disinfection outdoors with exposure to sunlight, and subsequently holding UV-treated wastewater effluent in a dark tank for 3 hours before release could lower *E. coli* photoreactivation rates.

### 4.4. Conclusion

The purpose of this study was to analyze *E. coli* concentrations in effluent collected after UV disinfection, investigate photoreactivation and dark repair rates, and determine any trends related to potentially pathogenic phylotypes. *E. coli* photoreactivation rates were higher than dark repair rates at 12 & 48 hrs. Potentially pathogenic phylotypes represented two-thirds of all *E. coli* sampled immediately after UV-irradiation treatment, which

adversely affects aquatic environments immediately downstream from WWTPs, especially if they are used for recreational purposes. Although potentially pathogenic phylotypes decreased in their proportion, they still represented a minimum of 10% of all *E. coli* phylotypes 48 hours later. Additionally, treated effluent kept in dark conditions showed a steady decrease in potentially pathogenic *E. coli* phylotypes. Results from this study suggest keeping UV-irradiated WWTP effluent in a dark environment for as long as possible before releasing it to downstream environments could lower *E. coli* concentration and potentially pathogenic *E. coli* phylotypes.

# CHAPTER V

# SUMMARY AND FUTURE RECOMMENDATIONS

## 5.1. Summary

Human and animal waste treatment/disposal systems aim to significantly reduce harmful pathogenic microorganism concentration before release to any surrounding environment. However, many animal waste systems are not closed systems and potentially pathogenic microorganisms, such as *E. coli,* can leach into nearby waterways. WWTPs also release potentially pathogenic *E. coli,* regardless of the final disinfection method. The two water segments examined in this study are in central Texas have been on the 303 (d) List of Impaired Waters for years due to elevated *E. coli* levels. One section was located downstream from dairy farms suspected to contribute to non-point source bacterial contamination. The other section was located downstream from two WWTPs, both known to release *E. coli* in treated effluent. Phylotyping *E. coli* strains isolated from multiple dairy farm manure management systems and from WWTPs gives insight into potential pathogenicity of *E. coli* released from both systems.

Phylotyping *E. coli* isolates from four separate dairy farms that employed different manure management systems (Chapter II) indicated that tiered manure management systems were more effective at reducing *E. coli* concentrations. Additionally, phylotype B1 was the most predominant phylotype.

Comparing *E. coli* phylotype distribution from two WWTPs using different disinfection methods (Chapter III) showed that the most dominant phylotypes pre-disinfection were A and B1, not B2 as previously thought. Post-disinfection the most dominant phylotype was B2 at the WWTP employing UV-irradiation. Although the QMRA for both WWTPs were not statistically different from each other, indicating almost equal levels of risk, a higher average portion of potentially pathogenic *E. coli* was observed at the WWTP employing UV-irradiation.

Studying *E. coli* photoreactivation and dark repair in WWTP effluent after UV-irradiation (Chapter IV) revealed decreasing concentrations of potentially pathogenic *E. coli* in samples kept in dark conditions. Immediately after UV disinfection potentially pathogenic *E. coli* phylotypes represented two-thirds of all isolates.

### 5.2. Future recommendations

Concerning dairy farm manure management practices, *E. coli* isolates from four dairy farms were phylotyped in this study. Multiple farms for each manure management practice, and additional locations around the state and nationwide would provide a more robust analysis of *E. coli* phylotype distribution at each step in the management practice. Additionally, sampling streams or rivers upstream and downstream from each farm could help determine if *E. coli* is being released and which management practice is contributing the highest proportion.

For the WWTP studies, I would recommend additional WWTP effluent sampled for each disinfection method, perhaps spread out through the state and nation. A comparative

analysis of *E. coli* concentration (and possibly general microbial diversity) upstream and downstream from each WWTP disinfection method. To more closely and accurately define photoreactivation and dark repair, I would recommend sampling WWTP effluent treated by UV-irradiation hourly for 12 hours, then every 2-3 hours up to 48 hours. Furthermore, isolating up to 20 *E. coli* colonies per treatment and replicate would give a more robust analysis. It would be beneficial to assess growth trends of each specific phylotype in a variety of media and a variety of treatments (dark and light, different temperatures, different surrounding microbial populations).

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